UNIVERSITY OF
LIVERPOOL

Influence of HIV and antiretroviral therapy on immunity to pneumococcal T-cell dependent antigens in Malawian adults

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of

Doctor of Philosophy

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July 2011

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DECLARATION OF WORK DONE

The work outlined in chapters 4 and 6 was part of two larger studies titled "**Pneumococcal immunity in Adults with HIV Infection** and '**Assessment of memory to the pneumococcus in HIV infected and uninfected Malawian adults following pneumococcal conjugate vaccination**'. The principal investigator for the two studies was Prof Rob S Heyderman and the principal supervisors were Dr S. Glennie and Dr Oluwadamilola H. Unuigbe-Iwajomo (PhD thesis, 2011). In some instances therefore work was shared among a number of individuals. My contributions for the reported work were as follows:

Activity	**Responsibility**
Sample processing	Shared
CFSE proliferation assay	Shared
Immunophenotying assay	Shared
CD154 expression assay	Sole
Intracellular cytokine staining assay	Sole
Flow cytometry data analysis	Sole
Statistical data analysis	shared
Thesis preparation	Sole

This work has not previously been accepted in substance for any degree and is not being currently submitted in candidature for any degree.

SIGNED: Enoch Sepako DATE: 15 - July - 2011

ABSTRACT

Background: Natural immunity to *S. pneumoniae* is thought to rely on antigen-specific T and B memory that can be rapidly mobilised to mediate microbial clearance at the mucosal surface as well as interrupt multiplication following invasion. HIV infection however increases the risk of invasive pneumococcal disease (IPD) and pneumococcal colonisation, suggesting that natural-acquired pneumococcal immunity is compromised in HIV infection. The aim of this research project was to investigate the effect of underlying HIV infection on naturally-acquired pneumococcal immunity in Malawian adults and the impact of antiretroviral therapy on this immunity. Additionally, the study evaluated the effect of HIV infection on immune memory mounted in response to vaccine protein antigens.

Methods: Peripheral blood mononuclear cells obtained from HIV-uninfected and infected Malawian adults were stimulated with pneumococcal concentrated culture supernatants derived from a standard strain D39 wild-type, isogenic mutant strain lacking pneumolysin (Ply-) and diphtheria toxoid. *In vitro* immune responses to these antigens were assessed by CFSE proliferation, T and B ELISpot, surface expression of CD154 and cytokine production by intracellular cytokine staining and bio-plex cytokine assays. Additionally, the proportion of T and B cell subsets including naive, memory, senescent and regulatory cells were determined using flow cytometry.

Results: Circulating central memory (T_{CM}) and naive (T_N) CD4+ T cells were preferentially lost in asymptomatic HIV infection. There was also an over representation of senescent and regulatory T cells in HIV-infected adults. Pneumococcal-specific immune responses were either compromised (IFN-γ effector responses, proliferative and CD154 expression) or altered (IFN-γ/IL-10) in asymptomatic HIV infection. HIV infection also increased the prevalence of nasopharyngeal colonisation in adults with advanced disease. There was some regeneration of pneumococcal specific CD4+ T cell responses including effector memory responses, proliferative capacity, CD154 pathway and ability to produce simultaneously multiple cytokines following initiation of ART. However, there was no regeneration of antigen-specific memory B cell responses. T and memory B cell responses to protein vaccine antigen (diphtheria toxoid) were compromised and appeared short-lived in HIV-infected persons well-established on ART.

Conclusion: The data from this research project showed that HIV infection compromises systemic naturally-acquired pneumococcal immunity and that there is some regeneration of this immunity following initiation of ART. It is not known however whether the re-constituted immunity is long-term and protective. The data also suggests that immune memory to vaccine protein antigens is compromised in HIV-infected adults and possibly short-lived even in individuals stable on ART. These findings have important implications for clinical care of HIV-infected persons on ART and future studies on pneumococcal immunity in the context of HIV infection and therapy.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors Professor R. Heyderman and Dr S Gordon for their guidance, helpful comments, support and keeping me focused throughout the three years of my studies. I thank also Drs S Glennie, Oluwadamilola H Unuigbe-Iwajomo and Kondwani C Jambo for providing some of the protocols and the reagents and for their helpful and invaluable discussions and contribution to this thesis. I extend my gratitude to everyone in the Tissue culture laboratory at Malawi -Liverpool Wellcome Trust Clinical Research Programme for sharing the space to carry out the work presented here and friendship throughout the years.

I especially want to thank my wife, Maipelo 'Mighty' for being the best wife there is. I am extremely grateful for all the understanding, patience and immeasurable support throughout the period of my studies. To my dad, mum, brothers and sister thank you for your love.

I would like to thank all the patients who participated in the different studies outlined in this thesis, without whom this work would have been impossible and the University of Liverpool for awarding me the studentship to pursue this PhD programme.

Finally, I would like to thank the almighty God for giving me the strength to come this far and keeping me healthy throughout the period of my studies. For I have always known that 'I can do all things through Him who strengthens me.' Philippians 4:13.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

CHAPTER 2 SUBJECTS, MATERIALS AND METHODS

CHAPTER 3 ASSAY OPTIMISATION AND ADAPTATION

LIST OF TABLES

ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APC	Allophycocyanin
ART	Anti-retroviral Therapy
ARV	Anti-retroviral
ASC	Antibody Secreting Cells
BAL	Bronchoalveolar lavage
BCG	*Bacille Calmette-Guérin*
BCIP	5-Bromo-4-chloro-3'-Indolyphosphate p-Toluidine salt
BD	Becton Dickinson
BFA	Brefeldin A
BSA	Bovine Serum Albumin
CbpA	Choline binding protein A
CBP	Choline binding protein
CCR7	Cysteine Chemokine Receptor 7
CDC	Centre for Diseases Control
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony forming Unit
CHI	Chronic HIV infection
COMREC	College of medicine Research and Ethics Committee
CMV	Cytomegalovirus
CRM_{197}	Cross-reactive material 197
CTL	Cytotoxic T lymphocytes
CVID	Common Variable Immunodeficiency
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DT	Diphtheria toxoid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-linked immunospot
FACS	Fluorescence activated cell sorter
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissue
HAART	Highly Active Antiretroviral Therapy
HBSS	Hank's Buffered Salt Solution
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HIV	Human Immunodeficiency Virus

ICS	Intracellular Cytokine Staining
IFN-γ	Interferon-gamma
IgA1	Immunoglobulin A1
IL	Interleukin
IQR	Interquartile range
IPD	Invasive Pneumococcal Disease
LRTI	Low Respiratory Tract Infection
LSTM	Liverpool School of Tropical Medicine
MFI	Mean fluorescence Intensity
MHC	Major Histocompatibility Complex
MLW	Malawi-Liverpool Wellcome Trust
MOH	Ministry of Health
MWCO	Molecular cut-off
NBT	Nitro-Blue Tetrazolium chloride
Nan	Neuraminidase
Nef	Negative factor
NIBSC	National Institute for Biological Standards and Control
OD	Optical density
PAFr	Platelet activating factor receptor
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCV	Polysaccharide conjugate vaccine
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PES	Polyethersulfone
PHA	Phytohemagglutinin
PiuA	Pneumococcal iron uptake
PneumoCCS	Pneumococcal concentrated culture supernatant
PMA	Phorbol myristyl acetate
PPD	Purified protein derivative
PPP	Purchasing power parity
PPV	Pneumococcal polysaccharide vaccine
PsaA	Pneumococcal surface Adhesion A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
PRR	Pathogen Recognition Receptor
PvA	Pneumococcal adhesion and virulence A
PWM	Pokeweed mitogen
QECH	Queen Elizabeth Central Hospital
RPMI media	Roswell Park Memorial Institute media
SAC	Staphylococcus aureus cowan
SEB	Staphylococcus enterotoxin B
SDS-PAGE	Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis

SFU	Spot Forming Units
SIV	Simian Immunodeficiency Virus
STGG	Skim milk-Tryptone-Glucose-Glycerol
TB	Tuberculosis
T_{CM}	Central memory T cells
T_{EM}	Effector memory T cells
T_N	Naive T cells
THY	Todd-Hewitt Yeast medium
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor- alpha
TT	Tetanus toxoid
UNAIDS	The Joint United Nations Programme on HIV/AIDS
UNICEF	United Nations Children's Fund
UK	United Kingdom
URT	Upper Respiratory Tract
USAID	United States Agency for International Development
VCT	Voluntary Counselling and Testing Clinic
WBC	White blood cell
WCV	Whole cell Vaccine
WHO	World Health Organisation

CHAPTER 1 Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae - the pneumococcus- is a Gram positive, encapsulated bacterial commensal that colonises the upper respiratory tract (nasopharyngeal cavity). It is believed that pneumococcal colonisation is mostly asymptomatic (Bogaert, De Groot et al. 2004), although recent data indicate that respiratory tract symptoms may occur (Sleeman, Daniels et al. 2005). Pneumococcal colonisation however can progress to respiratory or even systemic disease [Figure 1.1]. Pneumococcal disease will not occur without preceding nasopharyngeal colonisation with the homologous strain (Bogaert, De Groot et al. 2004). Pneumococcus causes life-threatening diseases such as pneumonia, septicaemia and meningitis (Cartwright 2002). In addition, it causes less serious but very prevalent diseases such as otitis media and sinusitis. Infection rates are high mainly in young children, the elderly, and individuals with immunocompromising conditions such as asplenia, cancer, chronic illness and HIV/AIDS (Jedrzejas 2001).

1.2 Pneumococcal Carriage and Risk factors

The upper respiratory tract is sterile prior to birth. The nasopharyngeal flora however is established within the first months of life (Bogaert, De Groot et al. 2004). In addition to bacteria that typically do not cause disease, a wide range of Gram-positive and Gram-negative bacteria that also have the potential to cause disease, such as *S. pneumoniae*, *H. influenzae, N. meningitidis* and *M. catarrhalis* can colonise the nasopharynx, albeit at various times throughout the host lifetime (Caugant, Hoiby et al. 1994; Adegbola, Mulholland et al. 1998; Harrison, Morris et al. 1999). Every person is likely to be colonised with these pathogens at least once during life and an individual bacterial strain can be carried for weeks to months before it is eventual cleared.

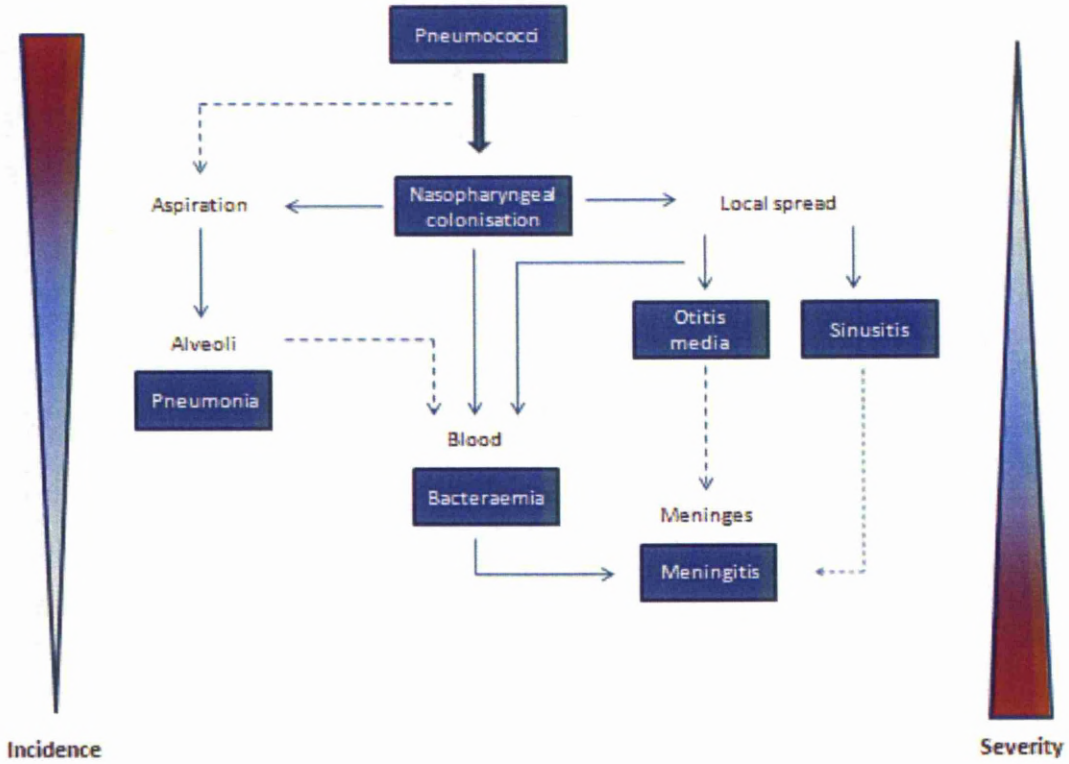


Figure 1.1 | Diseases caused by *Streptococcus pneumoniae*. Pneumococci colonise the nasopharynx, evade host immunity and spread to the middle ear, sinus, lower respiratory tract, blood and meninges. Pneumococci cause otitis media in the middle ear, sinusitis in the sinus, pneumonia in the lower respiratory tract, bacteraemia in blood and meningitis in the meninges. The incidences of different types of pneumococcal infection are inversely related to the severity of disease: otitis media is the most common but the least severe. *(Jambo, Sepako et al. 2010)*

With the pneumococcus, nasopharyngeal carriage prevalence is influenced by the age of individuals and the presence of pneumococcal carriers in the immediate environment (O'Brien, Dagan et al. 2008). Worldwide, carriage rates are highest among children under 2 years of age (Leino, Auranen et al. 2001; Regev-Yochay, Raz et al. 2004). Similarly, the prevalence of pneumococcal carriage in sub-Saharan Africa, is very high in early life (~70-90% of children aged <5 years) and continues well into adulthood (Hill, Akisanya et al. 2006).

Fifty to ninety percent of the children are colonised within months after birth (Hill, Akisanya et al. 2006; Granat, Mia et al. 2007; Rudan, Boschi-Pinto et al. 2008) and the cumulative frequency of colonisation in this group is almost 100% (Hill, Akisanya et al. 2006). For example, in the Gambia, the pneumococcus was acquired within the first months of life (93% < 1 month and 96% < 3 months) with the mean and median age at first acquisition of carriage noted as 33 days (95% confidence interval, 29–36 days) and 24 days, respectively (Rudan, Boschi-Pinto et al. 2008). In Malawi, the rate of pneumococcal colonisation was observed to be 84% among children less than 5 years of age, 65% of which occurred in children less than the age of 3 months (Feikin, Davis et al. 2003). Carriage rates however decrease significantly with increasing age (Hill, Akisanya et al. 2006). In the Gambia, the prevalence of pneumococcal carriage decreased to 51% among subjects > 40 years from 97% among children aged < 1 year ($p<0.001$). A similar situation was observed in Kenya, where among 0-4, 5-9 and 10-85 year old, pneumococcal carriage rate was 57%, 41% and 6.4% respectively (Abdullahi, Nyiro et al. 2008).

Data from this thesis (section 4.3.1) and other studies (French N, unpublished data) show that the prevalence of nasopharyngeal carriage among healthy Malawian adults (Blantyre district) is around 15%. Colonisation is generally followed by horizontal spread of *S. pneumoniae* to individuals in the immediate surroundings resulting in the spread of the pathogen within the community. Horizontal dissemination of pneumococcal strains is associated with risk factors such as overcrowding which is often seen in hospitals, day care centres, prisons and orphanages (Bogaert, De Groot et al. 2004). In a study in The Netherlands, there was a 1.6 fold relative risk of pneumococcal nasopharyngeal colonisation in children attending day care centres compared with children cared for at home (Bogaert, Engelen et al. 2001).

Similarly, a colonisation rate of up to 82% was reported in infants living in an orphanage in Paris, France (- a region of the world where carriage rates are generally lower) (Raymond, Le Thomas et al. 2000). In both The Netherlands and French studies, there was an increased genetic clustering of pneumococcal isolates, suggesting an increased and/or frequent horizontal spread of pneumococcal strains in crowded areas (Bogaert, De Groot et al. 2004).

1.3 Pneumococcal Disease in Africa

Childhood pneumonia is the leading cause of mortality in children less than five years of age in the world (Rudan, Tomaskovic et al. 2004; Rudan, Boschi-Pinto et al. 2008) and *S. pneumoniae* is one of the main pathogens associated with clinical pneumonia. In prospective microbiology-based studies, the pathogen was identified in 30-50% of pneumonia cases (Juven, Mertsola et al. 2000; Rudan, Tomaskovic et al. 2004). Berkley et al (Berkley, Lowe et al. 2005) noted that pneumococcus causes 25% of all preventable deaths in children less than 5 years of age and several other studies have shown that 60-90% of lower respiratory tract infections are caused by the pneumococcus (Cutts, Zaman et al. 2005; Levine, O'Brien et al. 2006).

The incidence estimate for children less than five years of age for developing countries is 0.29 episodes per child-year, which equates to 151 million episodes each year (Table 1.1) (Rudan, Tomaskovic et al. 2004; Rudan, Boschi-Pinto et al. 2008). Of all the new cases, 11-20 million (7-13%) cases are severe enough to require hospitalisation. An estimate of clinical pneumonia incidence for Africa is 0.33 episodes per child-year, translating into 35.33 million new cases per year (Table 1.1) (Rudan, Tomaskovic et al. 2004; Rudan, Boschi-Pinto et al. 2008). Africa has by and large, the highest burden of childhood mortality in the world and the continent accounts for 50% of worldwide deaths from pneumonia in children less than five years of age (WHO 2007).

There are no population-based data on the incidence of pneumococcal disease in adults in developing countries including those in Africa. A study of migrant workers in South Africa gold mines (high-risk group) in the 1970s revealed that one miner in eleven developed pneumococcal disease each year (Fedson and Scott 1999).

Fedson et al. estimated that approximately one in every 12-18 medical admissions in East and Southern Africa is caused by pneumococcal pneumonia (Fedson and Scott 1999). In a study

done in Soweto, South Africa, the annual incidence estimates for invasive pneumococcal disease for HIV-seronegative adults 18-40 and >65 years in age were 24 per 100,000 and 64 per 100 000 respectively (Jones, Huebner et al. 1998).

Table 1.1 | Estimates of incidence and number of new cases per year of clinical pneumonia in children aged less than 5 years, by WHO region[a] *(Rudan T et al. 2008)*

WHO region	Total population aged 0-4 years (millions)	Estimated Incidence (e/cy)	Estimated no. of new cases per year (millions)
African	105.62	0.33	35.13
Americas	75.78	0.1	7.84
Eastern Mediterranean	69.77	0.28	19.67
European	51.96	0.06	3.03
South-East Asia	168.74	0.36	60.95
Western Pacific	133.05	0.22	29.07
Total (developing countries)	523.31	0.29	151.76
Total (developed countries)	81.61	0.05	4.08
Total	604.93	0.26	155.84

e/cy, episodes per child-year.
[a] Up to 10% of all new cases may progress to severe episodes and require hospitalization.

1.4 HIV Epidemiology

Human immunodeficiency virus (HIV) and its associated syndrome, acquired immunodeficiency syndrome (AIDS) remains a major global health problem with millions of people especially in Africa either infected or affected by HIV/AIDS. Sub-Saharan Africa is the most affected region with an estimated 22.4 million [20.8 – 24.1 million] people living with HIV or 67% of the global total (UNAIDS, 2009) (Figure 1.2). HIV seroprevalence in many sub-Saharan African countries exceeds 10% overall, rising to over 30% in pregnant women.

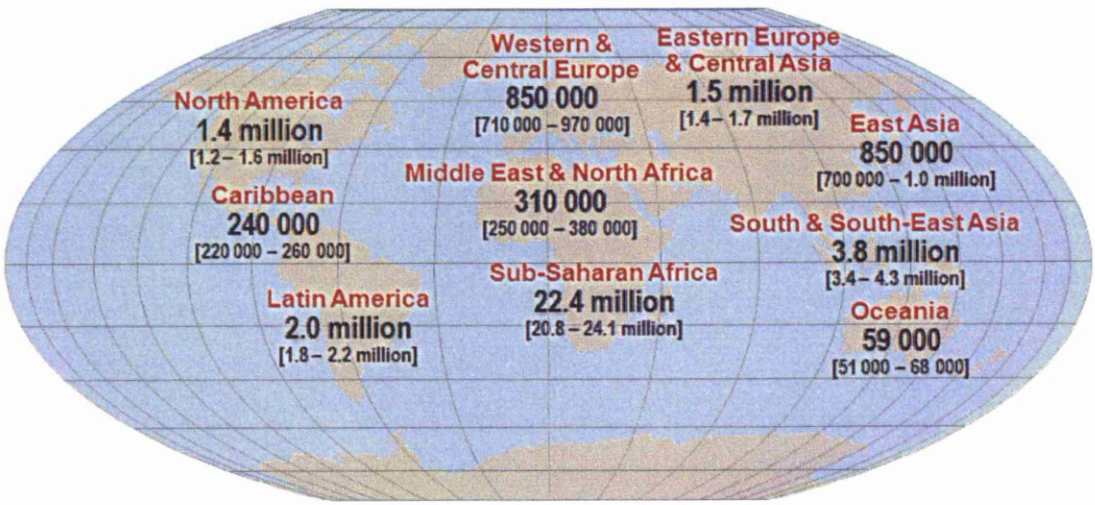


Figure 1.2 | Adults and children estimated to be living with HIV, 2008 *(UNAIDS, Aids epidemic update. 2009 [www.unaids.org])*

Increased carriage and susceptibility to disease by bacteria that normally colonise the upper respiratory tract (URT) is one of the earliest manifestations of HIV infection in both industrialised and non-industrialised countries (Manfredi, Nanetti et al. 2000; Pan, Diep et al. 2005).

1.5 Pneumococcal Carriage in the context of HIV

Data on pneumococcal nasopharyngeal carriage in HIV-infected persons suggests that carriage rates may be higher in HIV-infected individuals, than in uninfected persons (Rodriguez-Barradas, Tharapel et al. 1997; Madhi, Adrian et al. 2007; Gill, Mwanakasale et al. 2008). Patients with low $CD4^+$ counts were more likely to be persistent pneumococcal carriers (Rodriguez-Barradas, Tharapel et al. 1997), and HIV-infected children were observed to have a higher prevalence of pneumococcal colonisation than HIV-negative children (Madhi, Adrian et al. 2007). More recently, a study in Zambian mothers demonstrated that HIV infection increased the risk of colonisation, (risk ratio [RR], 1.9; 95% confidence interval [CI], 1.3-2.8) and repeat colonisation (RR, 2.4; 95% CI, 1.1-5.3), and reduced the time to new colonisation (P = .01)(Gill, Mwanakasale et al. 2008).

1.6 Pneumococcal Disease and HIV Infection

The emergence of HIV infection has dramatically changed the epidemiology of pneumococcal disease in both adults and children. The HIV pandemic has resulted in a large increase in the incidence and severity of childhood pneumonia in developing countries (Zar 2004). Data from a number of studies indicate that the incidence of hospitalisation for lower respiratory tract infection (LRTI) is higher in HIV-infected children than in their HIV uninfected counterparts (Mofenson, Yogev et al. 1998; Madhi, Petersen et al. 2000; Madhi, Kuwanda et al. 2005). The incidence of hospitalisation among children not vaccinated with a polysaccharide conjugate vaccine (PCV) in South Africa was almost 6.6 fold higher among HIV-infected children (16.7 cases for every 100 children per year) than in their HIV uninfected counterparts (2.6 cases for every 100 children per year) (Madhi, Petersen et al. 2000). In the United States, the incidence of pneumonia in HIV-infected children was found to be seven fold higher than the historical incidence rates of 3 to 4.2 cases for every 100 children per year (Foy, Cooney et al. 1973; Klugman 2008; Madhi and Pelton 2008). In some sub-Saharan African countries, approximately 45% of children hospitalized for pneumonia are HIV-infected (Madhi, Petersen et al. 2000).

HIV-infected individuals (adults and children) are 20 to 100 times more likely to suffer invasive pneumococcal disease (IPD) than their age-matched HIV negative persons (McEllistrem, Mendelsohn et al. 2002; Klugman 2008). Though highly active antiretroviral therapy (HAART) has been associated with a 60% reduction in IPD, the risk of disease in HIV positive persons is 30 times greater compared to uninfected individuals (Heffernan, Barrett et al. 2005). The burden of pneumococcal disease is HIV infected children in Africa is 40-fold higher than in developed countries (Klugman 2008). In South Africa, the rates of IPD in HIV-infected children under 2 years of age was found to be 42-fold (95% confidence interval 27-66) greater than that in HIV negative children (Klugman, Madhi et al. 2007) whereas in the USA the burden of the disease was 9-13 fold greater in HIV positive children pre-HAART era (Klugman, Madhi et al. 2007).

It is estimated that HIV accounts for at least 50% of all serious pneumococcal infections and pneumonia cases in adults in Africa (French 2003; Klugman, Madhi et al. 2007; Klugman 2008). A survey (case series) of 167 patients with IPD in Blantyre, Malawi, identified HIV infection as the most common risk factor for IPD (Gordon, Chaponda et al. 2002). Ninety-five percent of the patients suffering IPD were HIV-infected. Similar high rates of pneumococcal bacteraemia were recorded in the USA in the pre-HAART era (1094/ 100 000 in 1995/6) (Heffernan, Barrett et al. 2005; Klugman, Madhi et al. 2007).

1.7 Morphology, Cultivation and Identification of *S. pneumoniae*

Streptococcus pneumoniae cells are capsulated, gram positive, lancet-shaped cocci (one end is broad or rounded and the other is pointed). It was first isolated by George M. Sternberg and Louis Pasteur in 1880 (Austrian 1999). They usually occur in pairs (diplococci) with the rounded ends juxtaposed and the long axis of the coccus parallel to the line joining the pair (Paniker 2006). Pneumococci are characteristically small measuring about 1 μm in diameter.

Streptococcus pneumoniae is a fastidious bacterium, growing best in 5% carbon dioxide (microaerophilic cultivation). Nearly 20% of fresh clinical isolates require fully anaerobic conditions. *S. pneumoniae* is usually cultured in media that contain blood as growth requires a source of catalase (e.g. blood) to neutralize the large amount of hydrogen peroxide produced by the bacteria. In complex media containing blood, at 37°C, the bacterium doubles every 20-30 minutes (Howden 1976; Hansen 2000).

On blood agar, pneumococci grow as glistening colonies of about 1 mm in diameter. The colonies normally produce a zone of alpha (green) haemolysis (a greenish discoloration of the blood agar surrounding a bacterial colony or the darkening of the agar under the colony), which differentiates *S. pneumoniae* from the group A (beta haemolytic) streptococcus, but not from commensal alpha haemolytic (viridans) streptococci which are co-inhabitants of the nasopharyngeal cavity (Ryan 2004; Paniker 2006). Special tests therefore have to be carried out to differentiate the pneumococcus from *Streptococcus viridans* (Table 1.2).

In the laboratory, pneumococci are identified by sensitivity or susceptibility to optochin and to lysis by bile salts (deoxycholate) (Table 1.2). *S. pneumoniae* is sensitive or susceptible to optochin reagent while *Streptococcus viridians* is resistant (O'Brien and Nohynek 2003; HPA(UK) 2004; Paniker 2006). Additionally, *S. pneumoniae* produces a self-lysing enzyme (autolysin). Autolysin causes the organism, when grown in high density to undergo a characteristic autolysis that kills the entire culture. The presence of bile salt (deoxycholate) accelerates this process. A positive test appears as clearing of the culture's turbidity.

Table 1.2 | Differentiation between *Streptococcus pneumoniae* and *Streptococcus viridans*

	S. pneumoniae	*S. viridans*
Morphology	**Lancet-shaped**	**Oval or round cells**
	Capsulated	**Non-capsulated**
Optochin Sensitivity Test	**Sensitive**	Resistant
Blood -agar plates	Inhibition of growth	
Bile Solubility Test	**'Soluble'**	**Insoluble**
Broth culture	Clearing of the culture's turbidity	
Blood-agar plates	Colony 'disappearance'	
	Green zone of α-hemolysis unaffected	
Inulin Fermentation	**Not Ferment**	**Ferment**

1.8 Pneumococcal Virulence factors

1.8.1 Polysaccharide Capsule

Streptococcus pneumoniae virulence is multi-faceted. Essential pneumococcal virulence factors include: the capsule, cytoplasmic and cell-surface proteins (Figure 1.3). Nearly all strains of the pneumococcus have a polysaccharide capsule, which is the basis for serotyping (Lynch and Zhanel 2010). The capsule is the outermost layer of *S. pneumoniae* and except for serotype 3, it is covalently attached to the outer surface of the cell-wall peptidoglycan (Kadioglu, Weiser et al. 2008) . It is considered the most virulent part of the pneumococcus (Bergmann and Hammerschmidt 2006). Moreover, the virulence of the pneumococcus is related to the thickness of the capsule in a particular strain and serotype (Mac and Kraus 1950; Kadioglu, Weiser et al. 2008).

Over 90 distinct serotypes have been identified to date (Lynch and Zhanel 2010). However, a limited number of these serotypes (~20 serotypes) are responsible for over 80% of the global invasive pneumococcal disease burden (Lynch and Zhanel 2009). The most common disease-causing serotypes being 1,3,4,6A, 6B, 7F,8,9V,14,18C,19F and 23F. In young children, serotypes 6,14,18,19 and 23F predominate. Serotypes 1 and 5 are common in developing countries but are less common in industrialized countries reflecting differences in serotype distribution among geographic regions.

The capsule inhibits opsonisation of the underlying bacterial surface components and subsequent phagocytosis during invasion (Kadioglu, Weiser et al. 2008; Weiser 2009) and facilitates the organism's transit from the luminal mucus to the epithelial surface where stable colonisation occurs.

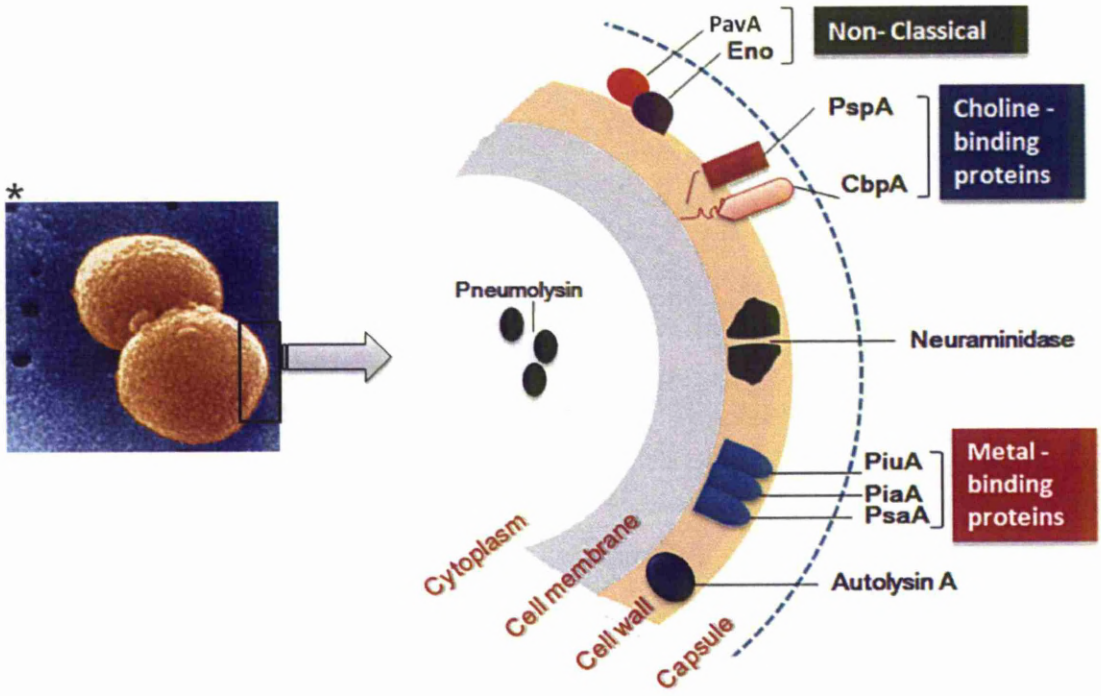


Figure 1.3 | Schematic diagram of pneumococcal virulence factors. *Streptococcus pneumpniae* synthesises several factors that are thought to have a role in pneumococcal disease process. These include the polysaccharide capsule, cytoplasmic and cell-surface proteins. PavA - pneumococcal adhesion and virulence A; Eno –enolase; PspA -pneumococcal surface proteins A;, CbpA – choline-binding protein A; PiuA - Pneumococcal iron uptake; PiaA - Pneumococcal iron acquisition and PsaA - Pneumococcal surface Adhesion A

**Image of S. pneumoniae from the Sanger Institute (www.sanger.ac.uk)*

1.8.2 Protein Virulence factors

1.8.2.1 Pneumolysin (cytoplasmic protein)

Streptococcus pneumoniae produces a single toxin, which is a member of the large cholesterol binding cytolysin expressed by gram-positive bacteria (Weiser 2009). Pneumolysin has a cytolytic and a noncytolytic activity (Preston and Dockrell 2008). The cytolytic activity is mediated by pore-formation and the noncytolytic activity is mediated arguably by complement activation and Toll-like receptor 4 binding (Preston and Dockrell 2008). Site-directed mutagenesis has shown that distinct parts of the toxin molecule are responsible for the two activities (Boulnois, Paton et al. 1991; Mitchell, Andrew et al. 1991).

Pneumolysin is cytotoxic to all cells with cholesterol in the membrane and as such interacts with the outer membrane of all eukaryotic cells (Cockeran, Anderson et al. 2002). The toxin alters and/or impairs both the function and viability of host cells even those required for clearing the pathogen (Weiser 2009). The cell-modulatory activities of pneumolysin are summarized in Table 1.3. By altering normal functioning of the immune cells pneumolysin may enhance persistence and invasiveness of the pneumococcus. In the case of alveolar epithelial cells and pulmonary endothelial cells for example, the toxin impairs the alveolar capillary barrier, triggering alveolar flooding and influx of nutrients for pneumococcal growth and facilitating penetration through respiratory epithelium into the blood stream (Cockeran, Anderson et al. 2002). The cell-modulatory activities of pneumolysin, somewhat paradoxically, may contribute to elimination of the *S. pneumoniae* by host defences. In a mouse model of pneumococcal colonisation it was observed that the interaction of pneumolysin and neutrophil promoted delivery and release of pneumococcal specific antigens to the nasal associated lymphoid tissues, enhancing adaptive immune responses against *S. pneumoniae* (Matthias, Roche et al. 2008; Jambo, Sepako et al. 2010).

Pneumolysin also activates the complement classical pathway. This appears to involve antibody binding (via Fc), by a region of pneumolysin homologous to C-reactive protein (a human acute-phase protein also capable of activating the classical pathway (Mitchell, Andrew et al. 1991). The interaction of pneumolysin with the classical pathway results in reduced C3 deposition on the pneumococcus, limiting opsonisation of pneumococci by the classical pathway (Yuste, Botto et al. 2005).

Animal studies show that pneumolysin is required for the development of pneumonia and bacteraemia. Data suggests that *S. pneumoniae* requires pneumolysin to successfully survive in both the upper and lower respiratory tracts (Berry, Ogunniyi et al. 1999; Orihuela, Gao et al. 2004; Kadioglu, Weiser et al. 2008) and as stated earlier pneumolysin is essential for the pneumococcus to translocate from the lungs to the bloodstream (Benton, Everson et al. 1995; Berry, Ogunniyi et al. 1999; Orihuela, Gao et al. 2004; Kadioglu, Weiser et al. 2008). It is also required for bacterial survival in blood. If a host is infected by a pneumolysin-expressing pneumococci not only are high numbers of bacteria detectable in blood but the host is rapidly overwhelmed by the infection (Benton, Everson et al. 1995; Berry, Ogunniyi et al. 1999). Conversely, in the case of pneumolysin negative pneumococci the animals have been shown to tolerate pneumococci with no obvious disease symptoms resulting in chronic bacteraemia (Benton, Everson et al. 1995; Kadioglu, Weiser et al. 2008).

Table 1.3 | Cell–modulatory activities of pneumolysin

Action	Cell-modulatory activity	References
Apoptosis	Induction of apoptosis in a number of cell types including neutrophils, macrophages & neuronal cells- related to influx of extracellular calcium into toxin-treated cells probably enhancing *S. pneumoniae* persistence and invasiveness	(Zysk, Bejo et al. 2000; Dockrell, Lee et al. 2001; Braun, Sublett et al. 2002)
Phagocytic function	Inhibition of the phagocytic functions of immune cells such as human neutrophil and monocyte respiratory burst	(Nandoskar, Ferrante et al. 1986) .
Chemotaxis	Stimulation CD4 T cell chemotaxis and recruitment of neutrophils to inflammatory lesion. Pneumolysin's complement-activating activity important for the recruitment of T cells while the toxin's cytolytic activity influences neutrophil recruitment	(Jounblat, Kadioglu et al. 2003; Hirst, Kadioglu et al. 2004)
Pro-inflammation	Induction of proinflammatory reaction in immune cells • production of IL-1β,TNF-α & NO by macrophages. The response involves signalling through Toll-like receptor 4 • Production of superoxide, prostaglandin E2, leukotriene B4 & elastase by neutrophils	(Cockeran, Anderson et al. 2002)

1.8.2.2 Cell-Surface Proteins

The surface of the pneumococcus is decorated with proteins that are covalently and non-covalently attached to the cell wall. Based on genomic analysis these proteins fall into at least three classes: the choline-binding protein (CBP) family, the lipoproteins and proteins that are covalently anchored in the cell wall by a LPxTG motif (a carboxy (c)-terminal sortase) (Kadioglu, Weiser et al. 2008). In addition to the three classes named above, there is a group of surface proteins (often referred to as non-classical proteins) that lacks a classical leader peptide and membrane anchoring motifs (Figure 1.3). Surface proteins either interact directly with the host tissues or are involved in masking the bacterial surface from the host defence mechanisms (Table 1.4).

1.8.2.2a Choline-Binding Proteins (CBPs)

The CBPs are a family of surface proteins non-covalently bound to the phosphorylcholine moiety of the cell wall of *S. pneumoniae* by a conserved choline binding domain consisting of 1 to 10 repeats of a 20-amino-acid sequence. Pneumococci can produce 13 to 16 different CBPs (Bergmann and Hammerschmidt 2006). Chief among the CBPs are pneumococcal surface protein A and C (PspA & PspC [CbpA]).

Pneumococcal surface protein A (PspA- protective antigen)

PspA (~92.5 kDa) is one of the most studied pneumococcal surface proteins because of its significant immune protective potential. It is relatively variable at the DNA and protein sequence levels and is expressed by almost all clinical serotypes (Briles, Hollingshead et al. 2000; Kadioglu, Weiser et al. 2008). PspA is known to play a key role in the pneumococcal virulence (Table 1.4). It inhibits complement deposition on the pneumococcal surface and thus prevents complement-mediated opsonisation of *S. pneumoniae* (Ren, Szalai et al. 2003; Yuste, Botto et al. 2005). In the absence of PspA function, pneumococcal virulence is attenuated and there is an increase in complement receptor- mediated clearance of pneumococci (Ren, Szalai et al. 2004).

PspA also binds the human iron transporter lactoferrin (van Rossum, Lysenko et al. 2005). The iron depleted form, apolactoferrin kills *S. pneumoniae* and as such the lactoferrin binding property of PspA may protect the pneumococcus from the bactericidal effects of apolactoferrin. In fact, Shaper *et al.* showed that PspA protects *S. pneumoniae* from killing by apolactoferrin (Shaper, Hollingshead et al. 2004). An additional property of PspA is its ability to inhibit the clearance of pneumococci from the bloodstream (Briles, Tart et al. 1998).

PspA has been shown to elicit an antibody response in children and adults over 50 years of age with pneumococcal disease (Rapola, Jantti et al. 2000; Samukawa, Yamanaka et al. 2000; Virolainen, Russell et al. 2000; Baril, Briles et al. 2004). During the course of the invasive disease, antibodies to PspA peak at the convalescent phase. PspA can also elicit T cell immune response in adults suffering invasive disease (Baril, Dietemann et al. 2006) and in such individuals both interleukin (IL)-10 and interferon (IFN)- production increased during convalescence suggesting that these cytokines may be involved in modulating antibody-based immunity to pneumococcal disease. In a mouse model, immunisation with PspA was highly immunogenic, eliciting IgG antibody in serum which correlated inversely with susceptibility to carriage (Quin, Moore et al. 2007).

Pneumococcal surface protein C (PspC [CbpA])

The pneumococcal surface protein C (PspC; also known as SpsA or CbpA ~ 75 kDa) is the largest and most abundant of the CBPs and is found in almost 70% of all pneumococcal strains. It is a multifunctional protein hence its several names. PspC is thought to facilitate the translocation of pneumococci across nasopharyngeal epithelial layer by binding the polymeric immunoglobulin receptor that normally transports secretory IgA (Zhang, Mostov et al. 2000). Furthermore, it interferes with the innate immune system by interacting with the complement factor H preventing C3b activation and complement-mediated opsonophagocytosis of pneumococci (Jarva, Hellwage et al. 2004).

Table 1.4 | Clusters of pneumococcal surface proteins and their role in pathogenesis

Cluster	Proteins	Role	Ref
Choline-binding proteins (CBP)	Pneumococcal surface protein A (PspA)	Binds iron transporter lactoferrin and inhibits complement deposition on the pneumococcal surface	(Ren, Szalai et al. 2003; Shaper, Hollingshead et al. 2004; Yuste, Botto et al. 2005)
	Pneumococcal surface protein (PspC)	Promotes uptake of pneumococci into epithelial cells. Interferes with the innate immune response against pneumococcus	(Zhang, Mostov et al. 2000; Jarva, Hellwage et al. 2004)
Lipoproteins (Metal binding proteins)	Pneumococcal surface Adhesion A (PsaA)	Substrate-binding lipoprotein of ABC-type manganese-transport system – a system which is involved in resistance to oxidative stress. Suspected to be an adhesin molecule	(Kadioglu, Weiser et al. 2008)
	Pneumococcal iron acquisition (PiaA) & Pneumococcal iron uptake (PiuA)	Iron-uptake ABC transporters. ABC transport system is involved in resistance to oxidative stress	(Kadioglu, Weiser et al. 2008)
LPxTG – anchored protein	NanA	Assist adherence by revealing host receptors and changes the surfaces of competing bacteria within the niche	(Burnaugh, Frantz et al. 2008; Weiser 2009)
Non-Classical	Pneumococcal adherence & virulence factor A (PavA)	Fibronectin adhesion	(Pracht, Elm et al. 2005)

IgA protease, Phosphorylcholine (Chop) and Autolysin

Human immunoglobulin A1 (IgA1), which is the predominant form of the IgA in the upper respiratory tract, classically works by blocking adhesive interactions of microbes with host tissues (Weiser, Bae et al. 2003). *Streptococcus pneumoniae* however expresses an IgA1 protease, an enzyme that cleaves human IgA1. Cleaved IgA1 enhances bacterial attachment to host cell (Weiser, Bae et al. 2003). Studies suggest that the cleavage of IgA results in formation of Fab fragments whose cationic charge neutralises the negative charge of pneumococcal capsule and enhances bacterial attachment to host cells (Weiser, Bae et al. 2003; Preston and Dockrell 2008).

Additionally, the interaction between IgA and IgA protease exposes phosphorylcholine (Chop), which mediates bacterial adherence to the epithelial cells via the platelet activating factor receptor (PAFr) and activate host cell signalling through this receptor (Kadioglu, Weiser et al. 2008; Preston and Dockrell 2008). Phosphorycholine (Chop) is a component of both cell-wall-associated acids and lipoteichoic acids and is present in many other microbes especially those that colonise the upper respiratory tract such as *Haemophilus influenzae*. It also anchors choline-binding binding proteins to the bacterial cell wall (Kadioglu, Weiser et al. 2008).

Autolysin is an enzyme that is capable of degrading the bacterial cell wall of an organism within which it is produced. The major autolysin of pneumococci is an amidase called LytA. This enzyme cleaves the N-acetyl muramoyl-L-alanine bond of pneumococcal peptidoglycan (Kadioglu, Weiser et al. 2008). It is thought that autolysin contributes to the virulence of *S. pneumoniae*, by mediating the release of pneumolysin from the pneumococcal cytoplasm during infection (Lock, Hansman et al. 1992). *S. pneumoniae* mutants lacking LytA, have been shown to have reduced virulence in mouse models of bacteraemia and pneumonia (Kadioglu, Weiser et al. 2008).

1.8.2.2b Lipoproteins

Pneumococcal lipoproteins play a major role in substrate transport and bacterial fitness. The pneumococcal surface adhesin A (PsaA~37 kDa) is common to all pneumococcal serotypes. It is the substrate-binding lipoprotein of an ABC-type manganese-transport system (Bergmann and Hammerschmidt 2006). It has been suggested that PsaA is an adhesin molecule because it has sequences that are also found in adhesin molecules from other streptococci (Kadioglu, Weiser et al. 2008). Mutations in PsaA cause pleiotropic effects (multiple effects from a single gene) including reduced adherence of pneumococci to host cells, attenuation in a mouse infection model, and hypersensitivity to oxidative stress (Marra, Lawson et al. 2002; Tseng, McEwan et al. 2002). Since PsaA mutants need manganese for normal growth, the pleiotropic effect may be a result of a deficient manganese transport system which in turn affect expression of other genes including adhesin (Kadioglu, Weiser et al. 2008).

Interestingly, anti-PsaA antibodies inhibit pneumococcal adherence to human nasopharyngeal epithelial cells and subcutaneous immunisation of mice with PsaA inhibits pneumococcal carriage (Johnson, Dykes et al. 2002; Romero-Steiner, Pilishvili et al. 2003). PsaA elicits antibody responses in children and adults over 50 years of age with pneumococcal disease (Rapola, Jantti et al. 2000; Samukawa, Yamanaka et al. 2000). In these studies, children at ages 6, 12, 18 and 24 produced antibodies to PsaA and the antibody concentration increased with age. In adults over 50 years, antibody concentration rose significantly during the course of the invasive disease.

Pneumococcal iron acquisition (PiaA) and Pneumococcal iron uptake (PiuA) are iron-uptake ABC transporter lipoproteins, and have been shown to be required for full pneumococcal virulence. A single mutation in either of the proteins have been shown to reduce virulence in models of pneumonia and bacteraemia while for a double mutant the attenuation was much more pronounced (Brown, Gilliland et al. 2002). Immunisation with PiaA and PiuA elicits protective antibodies that not only recognize these proteins but promote opsonophagocytosis of *S. pneumoniae* (Jomaa, Yuste et al. 2005).

1.8.2.2c Neuraminidase- LPXTG-anchored proteins

Neuraminidase (Nan) also known as sialidase is another virulence factor of *S. pneumoniae* that is present in all strains of pneumococci. The enzyme sequentially cleaves terminal sugars (sialic acid residues) common to many human glycoconjugates such as glycoproteins (Kadioglu, Weiser et al. 2008). The pneumococcus encodes at least three neuraminidase genes; *nanA, nanB* and *nanC* (Jedrzejas 2001), whereby most strains encode NanA and NanB and only 50% of the strains encode NanC.

The exact role of neuraminidase in pneumococcal pathogenesis is incompletely determined. However, in a murine model of acute pneumonia, NanA and B were shown to be essential for pneumococcal survival in the bloodstream and respiratory tract (Manco, Hernon et al. 2006). Others have shown that NanA removes sialic acid of human airway components (lactoferrin, secretory component, and IgA) that bind to the bacterial surface and possibly mediate bacterial clearance (King, Hippe et al. 2004). This effect may facilitate bacterial persistence in the respiratory tract (King, Hippe et al. 2004).

NanB is necessary for infection of lower and upper respiratory tract (Berry, Lock et al. 1996), and in the deglycosylation of host glycoconjugates, which *S. pneumoniae* utilises as a sole source of carbon to sustain growth (Burnaugh, Frantz et al. 2008). In a nutshell, deglycosylation of human glycoconjugates, may expose receptors on the cell-surface, prevent mechanisms of clearance that require these molecules or provide nutrients for bacterial growth (Weiser 2009). NanC is thought to have a tissue-specific role and was more common in isolates from cerebrospinal fluid than in carriage isolates (Pettigrew, Fennie et al. 2006).

1.8.2.2d Non-classical Proteins

There are a number of proteins that appear on the surface of *S. pneumoniae* but do not possess classical features of bacterial surface proteins. Pneumococcal adherence and virulence factor A (PavA) is one such protein. Studies suggest that PavA acts as a fibronectin adhesin and modulates vital but yet-to be determined virulence determinants (Pracht, Elm et al. 2005).

1.9 HIV Infection and Pathogenesis

Co-receptors and HIV pathogenesis

HIV infection of cells begins when the envelope glycoprotein (Env) of a viral particle binds to both CD4 and a coreceptor that is a member of the chemokine receptor family. In almost all cases, HIV uses chemokine receptors CCR5 and CXC4 as these co-receptors (Deng, Liu et al. 1996; Feng, Broder et al. 1996). CCR5 is expressed on a small fraction of memory CD4+ T cells, while the expression of CXCR4 is high on naïve T cells and decreases with differentiation and activation (Bleul, Wu et al. 1997; Gorry and Ancuta 2011). CCR5-using viruses (R5 viruses) are transmitted from person to person and are dominant in the early and chronic phases of HIV-1 infection (Keele, Giorgi et al. 2008). However, in roughly 40% to 50% of infected individuals, progression to late stages of infection is preceded by a switch in co-receptor usage, to viral variants able to use CXCR4 (X4 viruses) or both CCR5 and CXCR4 (R5X4 viruses) (Gorry and Ancuta 2011). The broadened cell tropism of X4 and R5X4 viruses, in part, contributes to more rapid disease progression in individuals who have a co-receptor switch. Nonetheless, most HIV-infected persons progress to the late stages of infection whilst exclusively harbouring R5 viruses.

Studies suggest that this R5 predominance is due to enhanced macrophage (M)-tropism, increased fitness, ability to scavenge low levels CD4 and CCR5 and resistance of CCR5 antagonist (Gorry and Ancuta 2011).

Role of DC in HIV infection

Dendritic cells (DCs) provide a highly specialised mechanism (through their antigen sampling and migratory capacities), whereby an antigen encountered in the peripheral tissues, notably skin and mucosal membranes, can be brought together with the T cells and in the process initiate adaptive immune responses against the antigen (Reis e Sousa 2006). HIV exploits this specialised mechanism to gain access to T cells in the lymph nodes, and establish a persistent infection of CD4+ T cells (Rowland-Jones 1999; Wu and KewalRamani 2006). At the mucosal surfaces, HIV infects or associates with immature dendritic cells (Burleigh, Lozach et al. 2006), but only the very early stages of viral replication occur in these cells. The maturing DCs then migrate to the lymphoid tissues that are enriched in CD4+ T cells. In these tissues, HIV infection of activated CD4+ T cells occurs, facilitating HIV dissemination (McDonald, Wu et al. 2003; Wiley and Gummuluru 2006; Wu and KewalRamani 2006).

The Course of HIV Infection

HIV infection is characterised by three phases namely the primary infection phase, the asymptomatic or chronic phase and the symptomatic phase or AIDS [Figure 1.4] (Centlivre, Sala et al. 2007; Douek 2007). The primary infection phase is associated with a substantial increase in plasma viral load and may be accompanied by a transient illness similar to glandular fever, with malaise, muscle pains, swollen lymph nodes, sore throat and rash (Roitt, Brostoff et al. 2001). However, the interaction between the virus and the host's immune system leads to a decrease of the viral load to a primary infection viral set point (Figure 1.4) (Douek 2007). Based on measurements in peripheral blood, during the primary infection phase there is a transient and often unimpressive decrease in $CD4^+$ T cell count (Centlivre, Sala et al. 2007). During this phase there is also a dramatic and a preferential depletion of $CD4^+$ memory T cells in mucosa especially in the gut-associated lymphoid tissue (GALT) which houses up to 80% of CD4+ T cells in the body (Centlivre, Sala et al. 2007; Douek 2007). This is maintained throughout the chronic phase.

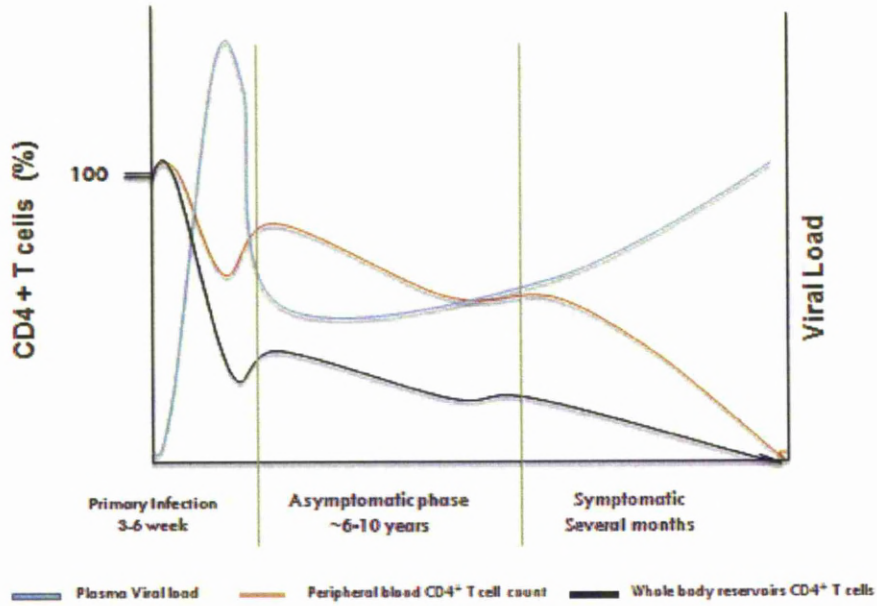


Figure 1.4 | Schematic diagram of a typical course of HIV-1 infection showing changes in plasma viral load, peripheral blood CD4 T –cell counts and whole body reservoirs of CD4+ T cells.

The GALT CD4+ T cells are depleted within 2 weeks of HIV infection (Sheth, Chege et al. 2008). The depletion of gastrointestinal CD4 T cells is associated with high frequencies of infected CD4 T cells (Brenchley and Douek 2008). Pulmonary mucosal site represented by BAL however appears to evade the massive CD4 T-cell depletion characteristic of the gastrointestinal tract.

The asymptomatic phase is characterised by a steady increase in viral load from the primary infection viral set point and a decrease in the number of $CD4^+$ T lymphocytes in peripheral blood (Figure 1.4) (Douek 2007). Symptomatic Phase or AIDS is associated with a terminal failure of the immune system (Douek 2007). The $CD4^+$ T cell numbers continue to fall and the viral load keeps rising (Figure 1.4). Additionally, non-specific constitutional symptoms such as fevers, night sweats and diarrhoea occur (Roitt, Brostoff et al. 2001). A variety of conditions that affect the mucous membranes and the skin such as oral candidiasis, shingles, and recurrent anogenital herpes simplex occur as well. These conditions are often a sign of the development of serious opportunistic infections and tumours such as *Pneumocystis carinii*, *Mycobacterium tuberculosis* and Kaposi's sarcoma (Roitt, Brostoff et al. 2001). These opportunistic infections usually constitute AIDS when the $CD4^+$ T cell count falls below 200 cells per microlitre.

HIV Infection and Immune Dysfunction

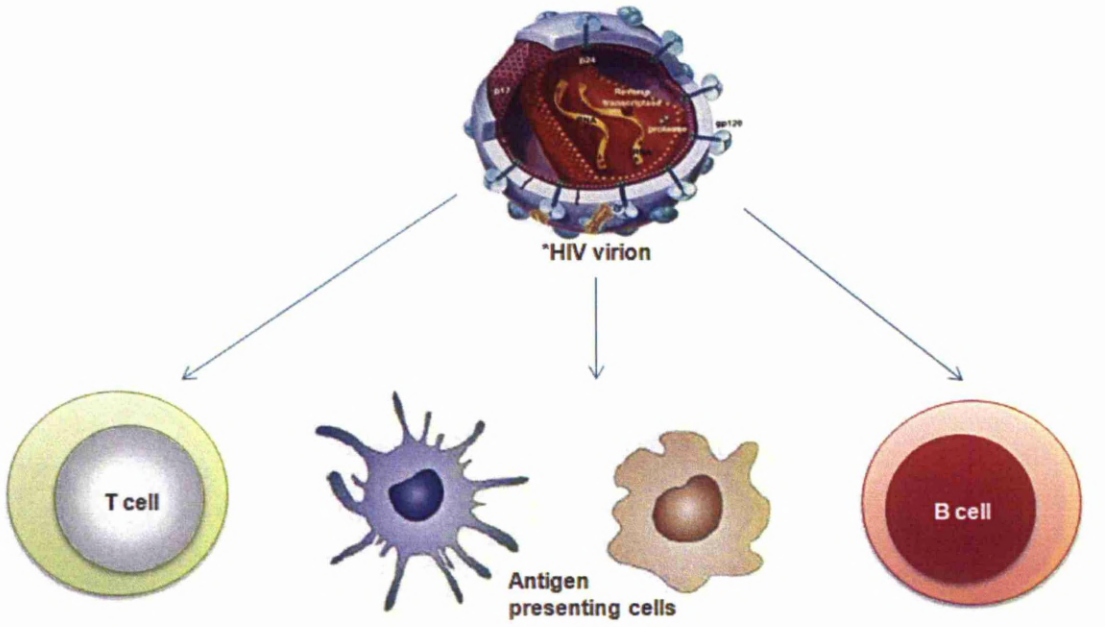
HIV infection is characterized by a generalized immune activation which affects almost all cell types in the immune system, causing severe immune dysfunction especially in the B and T cell compartments (Figure 1.5). Along with the general CD4+ T cell depletion, HIV infection causes a disproportionate depletion of Th17 cells in the gut and the peripheral blood of HIV-infected individuals (Brenchley and Douek 2008; Prendergast, Prado et al. 2010), which confer protection against extracellular and intracellular bacteria particularly at epithelial surfaces. There is also a preferential loss of resting naïve T cells during chronic stage, elevated levels of activated T cells (Douek 2007) and the inability of T cells to simultaneously produce effector cytokines (Betts, Nason et al. 2006).

In HIV-infected persons, HIV-specific CD4+ T cells are depleted before CD4+ T cells specific for other antigens (Douek, Brenchley et al. 2002; Yue, Kovacs et al. 2005). Studies suggest that preferential infection and preferential apoptosis may explain the preferential depletion of HIV-specific CD4 T cells in HIV-infected persons (Douek, Brenchley et al. 2002; Yue, Kovacs et al. 2005). Douek *et al.* showed that HIV-1 infects preferentially those $CD4^+$ T cells that are HIV-1 specific, rather than CD4+ T cells specific for unrelated (Douek, Brenchley et al. 2002), whileYue *et al.* showed that in HIV-1-infected individuals, a greater proportion of *ex vivo* HIV-1-specific CD4+T cells undergo apoptosis compared with cytomegalovirus (CMV)-specific CD4+ T cells (Yue, Kovacs et al. 2005).

Within HIV-infected individuals, different pathogen-specific CD4 T cells populations are differentially affected by HIV as well. A study looking at the depletion rates between CD4 T cells to two common opportunistic pathogens, CMV and *Mycobacterium tuberculosis* (*MTB*) revealed that CMV-specific CD4 T cells persisted after HIV infection, whereas *MTB*-specific CD4 T cells were depleted rapidly (Geldmacher, Ngwenyama et al. 2010). This is thought to be due to differences in function of the pathogen-specific CD4 T cells. CMV-specific CD4 T cells tended to produce MIP-1β but not IL-2, whereas *MTB*-specific CD4 T cells produced IL-2 but not MIP-1β (Geldmacher, Ngwenyama et al. 2010). In the same study, cells producing IL-2 in the absence of MIP-1β were more susceptible to productive HIV infection within SEB-activated CD4 T cells *in vitro*.

As stated above, HIV causes severe immune dysfunction in the B compartment. The precise mechanisms of HIV-mediated B-cell immunodeficiency are uncertain. As HIV is not known to directly infect B-cells, these defects could result from loss or dysfunction of CD4+ T cells or from high plasma viral loads resulting in immune activation and dysregulation (Moir, Malaspina et al. 2008). Direct B-cell dysfunction may result from the transfer of HIV negative factor (Nef) from HIV-infected macrophages to B-cells through long-range intercellular conduits (Xu, Santini et al. 2009). Hence while isotype-switched memory B cells may be sustained in HIV infection, their function is likely to be indirectly suppressed by HIV. Furthermore, B cells undergo phenotypic and functional changes, including depletion of memory B cell pools, which are responsible for the maintenance of serologic memory, hypergammaglobulinemia, impaired reactivity to immunization and loss of specific antibodies obtained as a result of normal vaccination (De Milito, Morch et al. 2001; De Milito 2004; De Milito, Nilsson et al. 2004; Titanji, De Milito et al. 2006; Cagigi, Nilsson et al. 2008)(Figure 1.5).

The memory B cell compartment is only partially restored by antiretroviral therapy (D'Orsogna, Krueger et al. 2007; Moir, Malaspina et al. 2008). HIV infection is also associated with numerous defects in the innate immune defences. Effector functions carried out by monocytes/macrophages are impaired by HIV infection, including phagocytosis, intracellular killing, chemotaxis and cytokine production (Kedzierska and Crowe 2002; Kedzierska, Azzam et al. 2003). The ability of macrophages to present antigens to T cells is also abnormal (Yoo, Chen et al. 1996) which may be due to decreased expression of MHC class II and B7 costimulatory molecules (Chougnet 2003). In addition, HIV infection is associated with depletion and impaired function of plasmacytoid and myeloid dendritic cells (DC) (Chougnet 2003; Reitano, Kottilil et al. 2009)



- Massive Depletion of mucosal CD4+ T cells[1]
- Altered production of effector cytokines [2]
- Decreased CD154 (CD40-Ligand) expression[3]
- Loss of Th17 cells[4]
- Inefficient CD8+ T cell activity[5]

- Defective phagocytic capacity[6]
- Impaired intracellular killing[6]
- Impaired chemotaxis[6]
- Altered cytokine and chemokine profile[6]
- Impaired DC maturation and function[6,7]
- Depletion of plasmacytoid and myeloid dendritic cells[8]

- Hypergammaglobulinemia[9]
- Increased polyclonal B-cell activation[10]
- Increased cell turnover[10]
- increased production of autoantibodies[10]
- Frequency of B-cell malignancies[11]
- Impaired reactivity to immunisation [12]
- Loss of vaccine-induced specific antibodies[12]
- Decline in total CD27 memory B cells[13]

Figure 1.5 | Direct and indirect effects of HIV on T, B cells and Antigen presenting cells (macrophages/monocytes and dendritic cells).

1. (Guadalupe, Reay et al. 2003; Brenchley, Schacker et al. 2004; Mehandru, Poles et al. 2004), **2.** (Chougnet and Gessani 2006), **3.** (Subauste, Subauste et al. 2007), **4.** (Prendergast, Prado et al. 2010), **5.** (Chougnet 2003), **6.** (Kedzierska and Crowe 2002; Chougnet 2003; Kedzierska, Azzam et al. 2003; O'Brien KL 2008), **7.** (Reitano, Kottilil et al. 2009), **8.** (Donaghy, Pozniak et al. 2001; Pacanowski, Kahi et al. 2001), **9.** (De Milito, Nilsson et al. 2004) **10.** (De Milito 2004; Moir and Fauci 2008; Moir and Fauci 2009), **11.** (Martinez-Maza and Breen 2002), **12.** (Bekker, Scherpbier et al. 2006), **13.** (De Milito, Morch et al. 2001; Titanji, De Milito et al. 2006). *HIV virion-adapted from (Abbas and Lichtman 2007).

1.10 Adaptive Immunity to extracellular pathogens

B cell-mediated (but not T cell-mediated) immune responses are the principal protective responses against extracellular bacteria as revealed by clinical observations of persons suffering from Bruton's agammaglobulinemia (Weiser and Nahm 2008). These individuals who have fairly normal T cell function but do not have mature B cells are particularly susceptible to bacterial infections caused by extracellular bacteria such as *Streptococcus pneumoniae,* a condition which can be effectively treated with passive administration of a broad spectrum of IgG antibodies (Lederman and Winkelstein 1985; Weiser and Nahm 2008).

During an infection, a large number of antigens such as capsular polysaccharides and proteins are shed by the bacteria. These antigens or their antibodies are often used to diagnose infections (Scott, Hannington et al. 1999; Weiser and Nahm 2008). Bacterial proteins elicit strong immune responses in a conventional T cell-dependent manner while bacterial polysaccharides generally stimulate antibody production in the absence of MHC class II restricted T cell help hence they are classified as T cell-independent antigens. Polysaccharides are able to stimulate antibody production because they usually have numerous repeating units and many epitopes which enable them to efficiently cross-link B cell receptors and stimulate B cells (Weiser and Nahm 2008). Polysaccharide antigens however are poor immunogens and generally do not stimulate the formation of germinal centres and easily tolerize B cells (Weiser and Nahm 2008).

The polysaccharides mainly stimulate two subsets of B cells: B1 B cells and marginal zone (MZ) B cells (Guinamard, Okigaki et al. 2000; Martin, Oliver et al. 2001; Weiser and Nahm 2008). B1 B cells, marginal zone B cells and follicular B cells are three subsets of mature B cells with preferential anatomic locations. B1 B cells are associated with the peritoneum, MZ B cells are found in the splenic marginal zone and follicular B cells are in the splenic follicles (Figure 1.6). Not all bacterial antigens are shed during an infection or when the bacteria enter the blood circulation. Some antigens remain attached to the bacteria. When the bacteria enter the blood circulation, they generally accumulate in the marginal zone of the spleen (Figure 1.6).

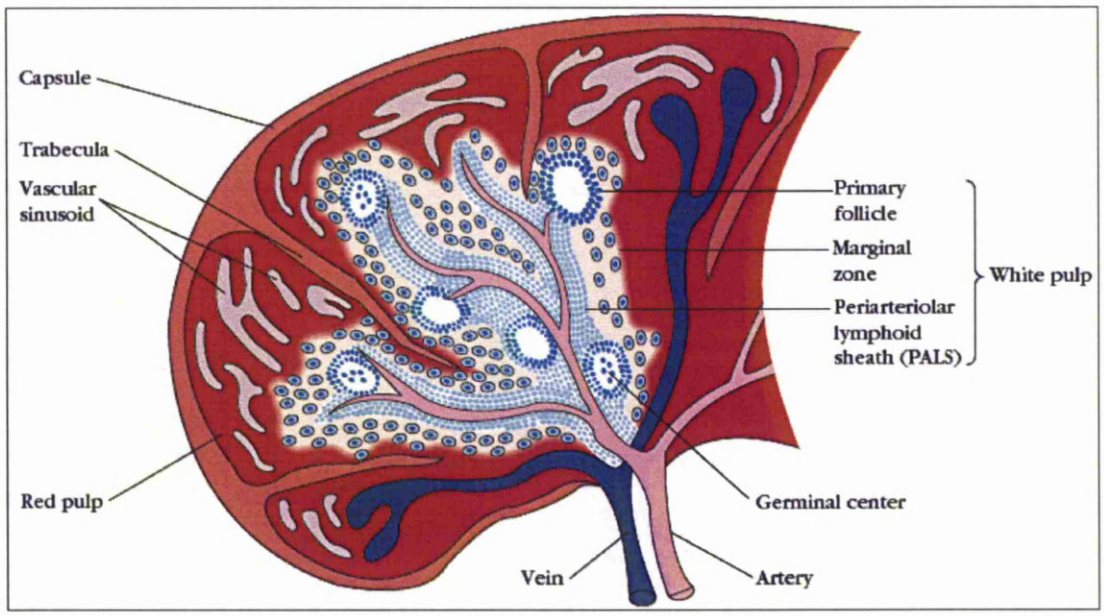


Figure 1.6 | Schematic diagram of the spleen. Microanatomically, the spleen has two types of compartments, the white pulp and the red (erythrocyte-filled) pulp. The white pulp forms a sleeve, the periarteriolar lymphoid sheath (PALS), around the arterioles; this sheath contains numerous T cells. Closely associated with the PALS is the marginal zone, an area rich in B cells that contains lymphoid follicles that can develop into secondary follicles containing germinal centres . Antigens are brought into the spleen along with arterial blood (Adapted from: (Goldsby, Kindt et al. 2003).

The marginal zone is best suited for capturing particles in the blood: the zone is where the terminal arteriole terminates and empties into sinuses and is filled with macrophages, dendritic cells (DCs) and pre activated B cells (MZ B cells) (Pillai, Cariappa et al. 2005; Weiser and Nahm 2008). Within 2-3 days of exposure to invasive microbes and even in the absence of T cell, MZ B cells can be activated and mature into plasma cells secreting antibodies (mainly IgM antibodies) to bacterial polysaccharides (Martin, Oliver et al. 2001). The marginal zone B cells by their unique features (high complement receptor 2 expression and multireactivity) are best suited to initiate a quick and an efficient response against the poorly immunogenic polysaccharide antigens. Furthermore, MZ B cells may also aid the activation of follicular B cells because they can capture IgM immune complex and transport it to follicular dendritic cells (Ferguson, Youd et al. 2004; Lopes-Carvalho, Foote et al. 2005; Weiser and Nahm 2008). They can also present bacterial (protein) antigens to naive T cells (Lopes-Carvalho, Foote et al. 2005).

The protein antigens of extracellular bacteria activate $CD4^+$ helper T cells, to produce cytokines that stimulate B cell responses (antibody production).

Antibodies use a number of effector mechanisms to fight infection including neutralisation, opsonisation and phagocytosis and activation of complement by the classical pathway (Abbas and Lichtman 2007)(Figure 1.7). Neutralisation is mediated by high-affinity IgG and IgA isotypes. These antibodies inhibit infectivity of microorganisms by blocking the binding of the organisms to cellular receptor. Phagocytes express receptors for the Fc portions of IgG antibodies (FcRs are also expressed on B cells). IgG isotypes opsonise (coat) the pathogens and promote their phagocytosis by binding to the Fc receptors on phagocytes. The binding of natural antibodies (either IgG subclasses or IgM) to microbial surfaces not only opsonises invading microbes but also activates complement via the classical pathway, a major effector of the humoral immunity.

Once the classical pathway is initiated, a cascade of events (the 'complement cascade') follows. In this case, the central protein of the complement system, C3 is cleaved, generating C3a and C3b, and bringing about a cascade of further cleavage and activation events. C3b binds to the surface of microbes, leading to greater internalisation by phagocytic cells by opsonisation.

Although, immunity against extracellular bacteria is clearly centred on the B cell responses, recent studies suggest additional roles for T cells in responses to extracellular bacteria and their products. For instance, studies of *Bacteroides fragilis* induced abscess formation indicate that *Bacteroides fragilis* zwitterionic capsular PS can be ingested and processed by antigen presenting cells using a nitric oxide-dependent mechanism and present it in association with MHC class II molecules to stimulate CD4+ T cells to produce IL-17 (Tzianabos, Finberg et al. 2000; Cobb and Kasper 2005; Weiser and Nahm 2008). Th17 CD4 T cells appear to confer protection against extracellular bacteria particularly at epithelial surfaces (Curtis and Way 2009) and studies in mice have shown that IL-17 receptor deficiency or deficiency of IL-23 is associated with increased risk of infections by extracellular bacterial infections (Happel, Dubin et al. 2005; Weiser and Nahm 2008).

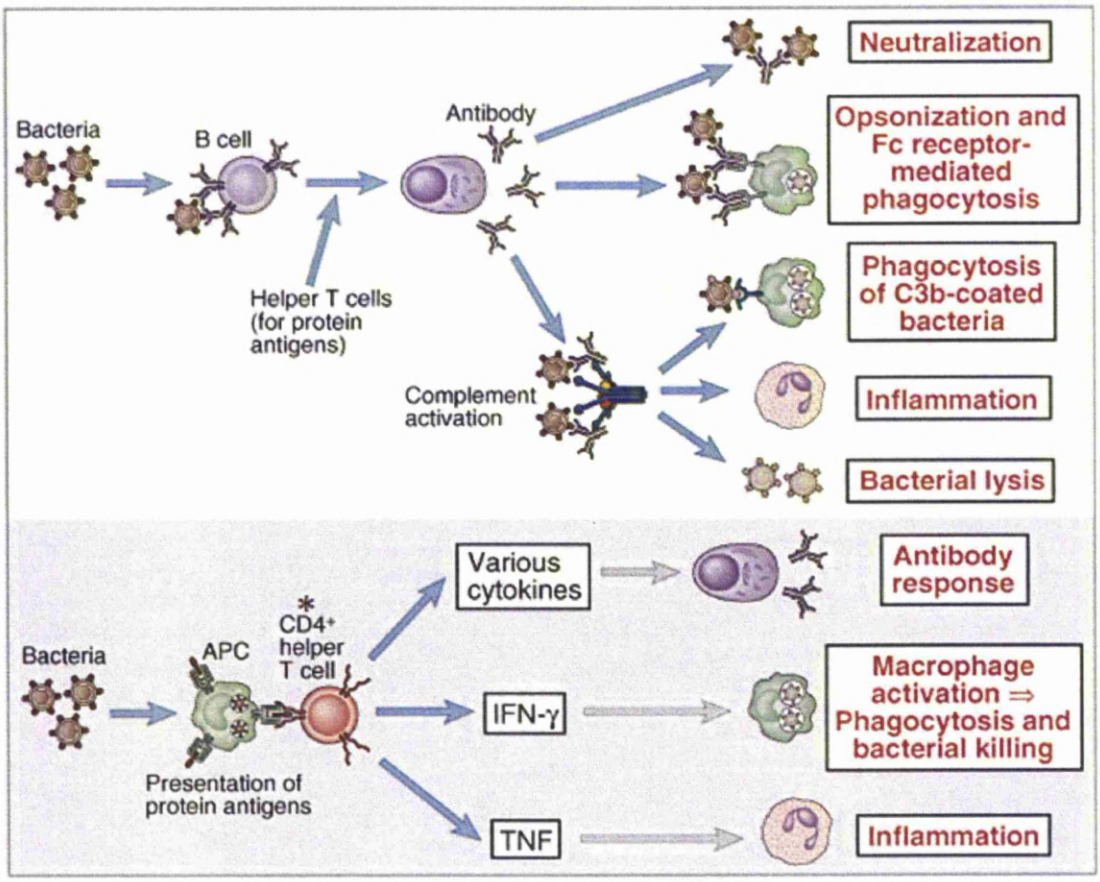


Figure 1.7 | Adaptive Immunity to extracellular bacteria. Adaptive immunity against extracellular bacteria is mediated primarily by antibodies. Antibodies protect against infection through a number of mechanisms including opsonisation and phagocytosis and activation of complement by the classical pathway. Extracellular microbes also activate CD4 T helper T cells to produce cytokines that stimulate B cell responses, macrophage activation (IFN-γ) and *neutrophil recruitment (IL-17). APC -antigen-presenting cell; IFN-γ- interferon-γ; TNF- tumor necrosis factor. Adapted from (Abbas and Lichtman 2007).

IL-17 has been shown to participate in host defence against extracellular pathogens, such as *Klebsiella, Candida* and *S. pneumoniae* in mice (Ye, Garvey et al. 2001; Huang, Na et al. 2004; Malley, Srivastava et al. 2006). The role of Th17 cells has been demonstrated in vaccine-induced immunity regardless of whether whole organisms, killed or live, were used. Animal studies showed that protection against *Bordetella pertussis* and *S. pneumoniae* (experimental pneumococcal colonization) with a whole cell vaccine induced Th17-dependent protection (Higgins, Jarnicki et al. 2006; Lu, Gross et al. 2008).

It appears IL-17 mediates its protective effects against extracellular bacteria by activating early neutrophil recruitment into local sites of infection (Curtis and Way 2009). The protein antigens of extracellular bacteria also stimulate $CD4^+$ helper T cells to produce cytokines that enhance the phagocytic and microbicidal activities of macrophages (Figure 1.7). Interferon-γ (IFN-γ) is the T cell cytokine responsible for macrophage activation.

1.11 Innate immunity to *S. pneumoniae*.

There is a huge disconnect between frequent rates of asymptomatic nasopharyngeal colonisation in humans and the relatively lower prevalence rates of invasive pneumococcal diseases (Greenwood 1999; Mulholland, Ogunlesi et al. 1999; Obaro and Adegbola 2002), suggesting the existence of naturally acquired protective immunity against pneumococcal disease. The development of natural immune response to pneumococcal infection generally involves both innate and adaptive immunity working in concert, to bring about protection against pneumococcal infection.

Innate immunity represents the first non-specific step in host defence and covers a wide range of host defences, including mucociliary clearance, complement, neutrophils and macrophages (Paterson and Mitchell 2006). Through a diverse array of mechanisms, such as phagocytosis, intracellular killing and activation of pro-inflammatory or antiviral cytokine production, the cells of the innate immune system trigger and support the adaptive immunity.

Complement

The complement system (over 30 serum and membrane proteins) is a biochemical cascade activated by three biochemical pathways (Walport 2001; Walport 2001; Paterson and Mitchell 2006): the classical pathway activated by antibody-antigen complexes; the lectin pathway, which is set off by mannose-binding lectin recognition of carbohydrate on the microbial surface and the alternative pathway, which is constantly triggered at low levels, but only amplifies on the surfaces of foreign particles. The role played by complement in innate and adaptive responses to pneumococcal infection is long established in humans; humans with complement deficiency are known to be at increased risk of invasive disease (Hostetter 2004). Studies in mouse models indicated that the classical pathway is the dominant pathway for innate immunity to the pneumococcus (Brown, Hussell et al. 2002).

It has been suggested that natural IgM antibodies and possibly acute-phase proteins such as C-reactive protein and complement component C1q contribute to the activation of the classical pathway (Paterson and Mitchell 2006; Rupprecht, Angele et al. 2007). The alternative pathway also contributes to protective innate responses, though to lesser degree compared to the classical pathway while the role of the lectin pathway is somewhat small (Paterson and Mitchell 2006).

Irrespective of the activation pathway, the deposition and activation of the complement component C3 on the bacterial surface is a critical step in the biochemical cascade which facilitates pathogen elimination. In line with the key role complement play in innate immunity, *S. pneumoniae* has developed ways of evading its effects. For example, the pneumococcal capsule, not only restrict access to cell-bound complement but also reduces the amount of complement that can be deposited on the bacterial surface (Abeyta, Hardy et al. 2003). Additionally, Pneumolysin activates the classical pathway by binding to the Fc region of immunoglobulin G (Paton 1996) and this interaction reduces C3 deposition on the pneumococcus, limiting opsonisation of pneumococci by the classical pathway (Yuste, Botto et al. 2005).

Innate immune cells

S. pneumoniae interacts with and activates phagocytic cells of the innate immune system including granulocytes, monocytes/macrophages and dendritic cells (DCs), thus indirectly stimulating the production and recruitment of T and B cell responses.

Pneumococcal colonisation of the murine upper respiratory tract triggers an acute inflammatory response characterized by the influx of neutrophils into the para-nasal spaces (Nelson, Roche et al. 2007) stimulating the production of inflammatory cytokines and chemokines. Pneumolysin stimulates neutrophil recruitment (van Rossum, Lysenko et al. 2005) and in a mouse model of pneumococcal colonisation the interaction of pneumolysin and neutrophils promoted delivery and release of pneumococcal specific antigens to the nasal associated lymphoid tissues, enhancing adaptive immune responses against *S. pneumoniae* (Matthias, Roche et al. 2008; Jambo, Sepako et al. 2010).

Vital components of the innate immune system are the so-called pathogen recognition receptors (PRRs). Their function is to detect microbes or pathogens and initiate the production of a variety of inflammatory mediators and cytokines and the phagocytosis of the invading pathogen (Henneke, Takeuchi et al. 2002). Among the key PRRs important in pneumococcal infection are members of the Toll-like receptor (TLR) family such as TLR-2 and TLR4 (Srivastava, Henneke et al. 2005; Dessing, Florquin et al. 2008). Antigen presenting cells such as dendritic cells, as well as macrophages, detect microbial infections by recognition via TLRs enhancing the production of pro-inflammatory cytokines and the up-regulation of MHC and co-stimulatory molecules (Colino and Snapper 2003).

Pneumococcal components have been found to be recognised by Toll-like receptor 2 (TLR2) and 4 (TLR4). TLR2 has been reported to influence pneumococcal disease progression (Srivastava, Henneke et al. 2005; Dessing, Florquin et al. 2008) and TLR2 knock-out mice have been shown to exhibit impaired pneumococcal clearance (van Rossum, Lysenko et al. 2005). Pneumolysin, a proven pneumococcal virulence factor interacts directly with TLR4 (Malley, Henneke et al. 2003; Zhang, Bagrade et al. 2007). The pro-inflammatory effect of pneumolysin on macrophages has been shown to be TLR4 dependent (Malley, Henneke et al. 2003), and macrophage apoptosis was found to regulate the clearance of bacteria in a murine model of pneumococcal pneumonia through the TLR4 ligand (Dockrell, Marriott et al. 2003).

In a pneumococcal colonisation mouse model, TLR4-deficient mice were more heavily colonised and more susceptible to infection compared to control mice (Malley, Henneke et al. 2003). In a human study, $CD4^+$ T cell proliferation induced by pneumolysin was inhibited by neutralising antibody to TLR4 but not TLR2 (Zhang, Bagrade et al. 2007). Thus, pneumolysin-specific $CD4^+$ T cell responses may be modulated by TLR4-dependent cellular activation probably of TLR4-expressing antigen presenting cells (APC) such as dendritic cells and macrophages (Beutler 2004).

1.12 Naturally-acquired immunity to *S. pneumoniae.*

In HIV-unaffected persons, as with many mucosal pathogens, pneumococcal disease becomes less frequent with age, a process which is thought to be due to the acquisition of natural adaptive immunity. In most individuals, programming of the immune system occurs as a result of carriage of the pneumococcus and related bacteria in the nasopharynx (Rapola, Jantti et al. 2000; Soininen, Pursiainen et al. 2001; Zhang, Choo et al. 2002; Heyderman, Davenport et al. 2006; Zhang, Bernatoniene et al. 2006; Zhang, Bagrade et al. 2007). The characteristics of the immunity involved in this upper respiratory tract (URT) process are not well understood. Natural immunity to the pneumococcus had been thought to be largely mediated by anti-capsular antibodies (Lipsitch, Whitney et al. 2005). This assumption was based on a number of observations.

In unimmunised populations, the incidence of invasive disease follows a familiar age distribution pattern. It peaks in the first 2 years of life and drops by more than an order of magnitude by the second and third decades of life. Thereafter, the incidence goes up quickly, with incidence in individuals over 70 years nearly the same as that in infants (Robinson, Baughman et al. 2001; Lipsitch, Whitney et al. 2005)]. Although the reason for the decline has not been fully established, it is frequently proposed that anti-capsular antibodies are involved (Musher, Groover et al. 1993; Lipsitch, Whitney et al. 2005).

Furthermore, data from several surveys show that carriage rates decline from over 50% in infants to 5-10% in adults and this correlate with a rise in both mucosal and serum anti-capsular antibody level (Gwaltney, Sande et al. 1975; Syrjanen, Kilpi et al. 2001; Simell, Kilpi et al. 2002; Zhang, Arnaoutakis et al. 2004; van Rossum, Lysenko et al. 2005). In a longitudinal household study, Goldblatt *et al.* reported a significant increase in anti-capsular IgG levels after carriage of serotypes 9V, 14, 18C, 19F, and 23F by an individual or a family member. For serotype 14, a higher level of anti-capsular IgG at the beginning of the study correlated with diminished odds of carriage, suggesting that naturally induced anti-capsular IgG can prevent carriage (Goldblatt, Hussain et al. 2005).

Passive transfer of anti-capsular antibody and polysaccharide-based vaccine studies show that anti-capsular antibodies are clearly sufficient to protect against invasive pneumococcal disease and colonisation (Malley 2010).

Immunisation with conjugate pneumococcal vaccine (7-valent and 9-valent vaccine), lead to a decrease in nasopharyngeal carriage of vaccine serotypes (Mbelle, Huebner et al. 1999; Huang, Hinrichsen et al. 2009) and the number of new acquisitions of nasopharyngeal pneumococcus (serotypes 14 and 19F) was inversely correlated with the serum serotype-specific pneumococcal anti-capsular IgG concentrations (Dagan, Givon-Lavi et al. 2005). An analysis of longitudinal carriage data from Israeli children in day care showed that some serotypes generate anti-capsular antibodies that can reduce the risk of carriage in unimmunised toddlers (Weinberger, Dagan et al. 2008; Malley 2010).

In addition to their serotype-specific polysaccharides, pneumococci express a range of sub-capsular antigens common among serotypes as described earlier in this chapter. Animal models and studies of human carriage have implicated some of these antigens in protection against both nasal colonization and invasive disease (Rapola, Jantti et al. 2000; Simell, Korkeila et al. 2001) (Balachandran, Brooks-Walter et al. 2002; Zhang, Choo et al. 2002; Zhang, Bernatoniene et al. 2006)(Table 1.5). Although, it is well established that anti-capsular and anti-subcapsular antibodies can protect against pneumococcal colonisation and disease it is less clear whether these antibodies are necessary for the natural development of protection against pneumococcal colonisation and disease (Malley 2010).

Data from sero-epidemiologic studies from the United States, Finland and Israel showed that unimmunised children were significantly less susceptible to pneumococcal invasive disease prior to the development of measurable systemic anticapsular antibodies. (Lipsitch, Whitney et al. 2005; Malley, Trzcinski et al. 2005). The age–specific decline in pneumococcal invasive disease was observed across serotype, arguing for a common and possibly a capsular serotype-independent mechanism of protection. Similarly, the reduction in the duration of carriage preceded by several years the detection of naturally-acquired anti-capsular antibodies in most children (Hogberg, Geli et al. 2007).

A longitudinal study in Bangladeshi children <1 year old, revealed that the rate of acquisition of colonisation declined in a manner consistent with the acquisition of immunity other than anti-capsular antibodies (Granat, Ollgren et al. 2009). In an experimental human carriage study, an increase in the antibody levels (serum and mucosal antibody response to capsular polysaccharide and PspA) did not correlate temporally with the loss of carriage, suggesting

that antibodies may play a limited role in the clearance of a pre-established colonisation (McCool, Cate et al. 2002; McCool, Cate et al. 2003; van Rossum, Lysenko et al. 2005).

Table 1.5 | Antibody responses to pneumococcal non-capsular antigens

Year	Antigen	Response	Human or Murine	Model	Ref
2000	PsaA, PspA & PdB	Serum IgG	Human	Natural carriage	(Rapola, Jantti et al. 2000)
2000	PsaA	Serum IgG	Human (infants)	Natural carriage	(Obaro, Adegbola et al. 2000)
2002	PspA	Serum IgG Secretory IgA	Human	Experimental carriage	(McCool, Cate et al. 2002)
2003	PspA & CbpA	Serum IgG	Human	Experimental carriage	(McCool, Cate et al. 2003)
2005	PsaA, PspA & Ply	Serum IgG	Human	Natural carriage (Longitudinal study)	(Goldblatt, Hussain et al. 2005)
2005	PsaA, PspA, PspC & PdB	Serum IgG Nasal secretion IgA	Murine	Experimental carriage	(Palaniappan, Singh et al. 2005)
2006	CbpA, PsaA, PspA & Pneumolysin	Serum & salivary IgG Adenoidal IgA, IgG & IgM	Human (children)	Natural carriage *In vitro* cell stimulation	(Zhang, Bernatoniene et al. 2006)

Studies in mice suggest that T cells play a role in the development of natural immunity to the pneumococcus (Malley, Trzcinski et al. 2005; Trzcinski, Thompson et al. 2005; van Rossum, Lysenko et al. 2005; Basset, Thompson et al. 2007; Trzcinski, Thompson et al. 2008). Murine challenge models using B cell, T cell, β2-microglobulin and MHC Class II knockouts suggest that immunity to pneumococcal colonisation and infection is antibody independent but is dependent on the presence of $CD4^+$ T cells at the time of challenge (Malley, Trzcinski et al. 2005; Trzcinski, Thompson et al. 2005; van Rossum, Lysenko et al. 2005). This CD4+ dependent immunity is mediated by interleukin-17A in a neutrophil-dependent manner (Lu, Gross et al. 2008). Pneumococcal specific-Th17 cells have been detected in adults and children, but not in newborn babies, suggesting that Th17 may be a result of natural exposure to *S. pneumoniae* (Lu, Gross et al. 2008).

Mucosal $CD4^+$ T cellular immunity to pneumococcal protein antigens has been shown in children and seems to modulate nasopharyngeal carriage (Zhang, Bagrade et al. 2007). In the same study, systemic $CD4^+$ T cellular immunity to pneumococcal protein antigens was demonstrated as well and this naturally acquired immune memory persists into adulthood (Zhang, Bagrade et al. 2007; Mureithi, Finn et al. 2009). The induction of CD4 T cell responses was associated with Th1-type cytokine production (IFN-γ, TNF-α, IL-12[p40]) and Th17-type cytokine production (IL-17, IL-12[p40]) (Zhang, Bagrade et al. 2007; Mureithi, Finn et al. 2009).

1.13 Vaccine prevention of pneumococcal disease- current strategies.

The first pneumococcal vaccine was a killed whole pneumococci vaccine and was introduced in 1911 by Sir Almroth Wright. Unfortunately, this vaccine failed as only one of the two serotypes identified was included and maximal vaccine dosage was inadequate (Watson and Musher 1999). Subsequently, vaccines based on capsular polysaccharide have been the main focus of pneumococcal vaccine research and implementation for many years (French 2003; Whitney 2005; Tai 2006) Tai SS et al. 2006). There are two formulations of pneumococcal vaccines that have been licensed thus far: pneumococcal polysaccharide vaccines (PPV) was developed first, followed by pneumococcal capsular polysaccharide-protein conjugate vaccine.

1.13.1 Pneumococcal Polysaccharide Vaccines

Capsular polysaccharides vaccines are purified capsular polysaccharides made from a selection of pneumococcal serotypes based on the relative frequency of serotypes in naturally occurring pneumococcal infections, aiming at a worldwide coverage of >90% of isolates that cause invasive in disease (Bernatoniene and Finn 2005). The first commercially available 14-valent polysaccharide vaccine was licensed in 1977 and by 1981 this was modified to a 23-valent vaccine (CDC[MMWR] 1984) (Figure 1.8). Polyvalent polysaccharides vaccines are efficacious in healthy adults. The 23-valent vaccine confers 60-80% protection against invasive pneumococcal disease in adults. However, it is poorly immunogenic in children <2 years of age, the age group which is most vulnerable to pneumococcal disease (Lynch and Zhanel 2009) and shows no protective effect against carriage (Obaro and Adegbola 2002). Additionally, it is less effective in HIV-infected adults and is not recommended for use in Africa (French, Nakiyingi et al. 2000; WHO 2008c).

In parts of the world, where the 23-valent vaccine is used, it is recommended for use early in the course of HIV disease (Masur, Kaplan et al. 2002). Poor immunogenicity of polysaccharide vaccines may be due to lack of T cell involvement which is a requirement for an increased antibody response in infants, induction of memory cells, and a booster response on subsequent antigenic exposures (Regev-Yochay, Malley et al. 2008).

1.13.2 Pneumococcal Conjugate Vaccines

Failure of polysaccharide vaccines to provide protection to those at the greatest risk, demonstrated the need to develop vaccines with expanded coverage and immunogenicity. The success of *Haemophilus influenza* type b (Hib) conjugate vaccine (Adegbola, Mulholland et al. 1998; Adegbola, Usen et al. 1999) and the *Neisseria meningitidis* serogroup C (Men-C) conjugate vaccine (Lakshman, Jones et al. 2001; Zhang, Lakshman et al. 2001; Lakshman and Finn 2002) led to the development of pneumococcal conjugate vaccines. In pneumococcal conjugate vaccines, a polysaccharide antigen is coupled to a protein carrier (e.g. diphtheria protein CRM_{197} or toxoid, tetanus toxoid or meningococcal outer-membrane protein) and the resulting conjugate acts as a typical T-cell-dependent antigen which is antigenic in infants.

T cells exposed to peptides derived from the protein carrier promote robust antigenic-specific B cell proliferation, affinity maturation and immunological memory (Mulholland, Hoestermann et al. 1994; Regev-Yochay, Malley et al. 2008). Several vaccine formulations incorporating between 4 to 13 pneumococcal capsular polysaccharides types have been investigated for safety and immunogenicity.

The seven-valent pneumococcal conjugate vaccine (PCV7) was licensed in the USA in the year 2000 and in Europe in 2001. The vaccine targets the seven serotypes that most often cause invasive pneumococcal disease (IPD) in children. (Figure 1.8) (Pavia, Bianco et al. 2009). Vaccination with PCV7 has resulted in a decrease in the incidence of IPD, pneumonia, bacteraemias and pneumococcal meningitis in adults and children (Table 1.6) (Adam 2009; Lynch and Zhanel 2010). In a meta-analysis of nine clinical trials of PCV7 in children < 2 years old, IPD caused by vaccine-serotypes and all serotypes fell by 89% and 63-74% respectively (Pavia, Bianco et al. 2009).

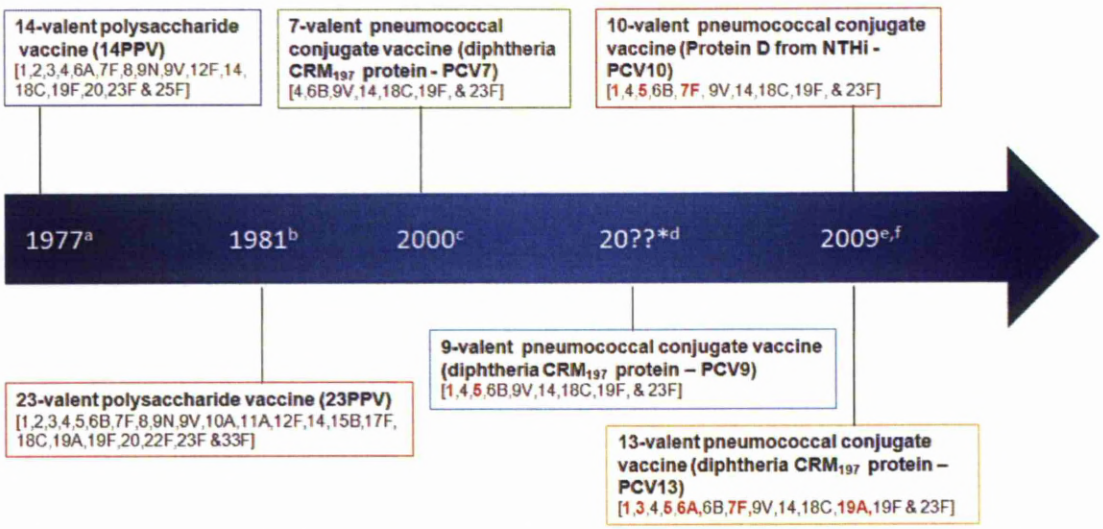


Figure 1.8 | Timeline- Highlights of development of pneumococcal vaccines. a,b|CDC[MMWR] 1984; c| Pavia, Bianco et al. 2009; d|Klugman, Madhi et al. 2003; e,f|*www.gsk.com/media/.../2009/2009_pressrelease_10039.htm* & Reinert, Paradiso et al. 2010. 1-additional serotypes to PCV7. **??*** - No date of licensure, withdrawn by manufacturer.

Table 1.6 | Outcomes of pneumococcal conjugate vaccines (PCV7 and PCV9)

Vaccine	Outcomes	References
Seven-valent pneumococcal conjugate vaccine (PCV7)	↓ vaccine-type IPD in children	(Whitney, Pilishvili et al. 2006)
	↓ mortality linked to invasive disease	(Tsigrelis, Tleyjeh et al. 2008)
	↓ nasopharyngeal carriage in children	(Ghaffar, Barton et al. 2004; Millar, O'Brien et al. 2006)
	↓vaccine-serotype carriage and IPD in unvaccinated children and adults (herd immunity)	(Flannery, Schrag et al. 2004; Lexau, Lynfield et al. 2005; Millar, Watt et al. 2008)
	↓ carriage & frequency of IPD cause by antibiotic –resistant pneumococci	(Kyaw, Lynfield et al. 2006)
	• Effective in HIV+ adults	(French, Gordon et al. 2010)
	§ Replacement by non- vaccine serotypes (particularly 19A) & ↑ non-vaccine type disease	(Pelton, Loughlin et al. 2004; Hicks, Harrison et al. 2007; Moore, Gertz et al. 2008; Dagan 2009)
Nine-valent pneumococcal conjugate vaccine (PCV9)	↓ incidence of radiologically confirmed pneumonia	(Klugman, Madhi et al. 2003; Cutts, Zaman et al. 2005)
	↓ vaccine-type & antibiotic-resistant IPD Among HIV-ve and HIV+ve children	(Klugman, Madhi et al. 2003)
	§ No herd immunity	(Cheung, Zaman et al. 2009)
	§ Replacement by non-vaccine serotypes	(Cheung, Zaman et al. 2009)

Additionally, vaccination protects unvaccinated population including adults reflecting herd immunity (CDC(MMWR) 2005). Recently, the vaccine was shown to protect HIV-infected adults from recurrent pneumococcal infections caused by vaccine serotypes or serotype 6A (French, Gordon et al. 2010). Post-licensure epidemiologic surveillance however show a replacement of the vaccine-serotypes with the non-vaccine serotypes, resulting in an increased incidence of pneumococcal disease caused by non-PCV7 serotypes (particularly serotype 19A) (Dagan 2009). Furthermore, PCV7 does not induce immunity to serotype 1 or 5, which are important causes of pneumococcal disease in many non-industrialised countries. Serogroup data from Malawi suggest that PCV7 would cover 41% of invasive isolates from children and 25% from adults (Gordon, Kanyanda et al. 2003). There are also indications that the vaccine may be less effective in overcrowded settings such as day care centres where pneumococcal infections are stubborn and persistent (Adam 2009).

A 9-valent conjugate vaccine which included serotypes 1 and 5 in addition to PCV7 (Figure 1.8), was successfully trialled in the Gambia (Cutts, Zaman et al. 2005) and South Africa (Klugman, Madhi et al. 2003) but was subsequently withdrawn by the manufacturer. The vaccine was effective in reducing mortality in the two populations. However, there was no evidence of herd immunity in the Gambia, where pneumococcal carriage rates are very high (Cheung, Zaman et al. 2009). Two new vaccines were licensed in Europe in 2009, the 10-valent and 13 valent pneumococcal conjugate vaccines (Figure 1.8). The 10 valent vaccine includes three additional serotypes (1, 5 and 7F) (Vesikari, Wysocki et al. 2009; Wysocki, Tejedor et al. 2009) which are highly invasive and increasingly prevalent (representing 5-25% of all IPD cases in children) (Munoz-Almagro, Jordan et al. 2008). The 13-valent vaccine is a response to the changing epidemiology of pneumococcal serotypes in countries where there is general or widespread use of PCV7 and to the increasing disease burden by serotype 19A. The vaccine includes additional six serotypes (including 19A) to PCV7 (Reinert, Paradiso et al. 2010). It is predicted that PCV13 will increase the percentage of vaccine-preventable cases of IPD to up to 90% or more (based on serotype data available before wide or general use of PCV7) in many parts of the world (Reinert, Paradiso et al. 2010).

1.13.3 Whole Cell Vaccine

Whole-cell vaccine (WCV) is usually made from ethanol-killed (70% (vol/vol) ethanol at 4°C) cells of a noncapsulated strain of *Streptococcus pneumoniae.* A noncapsulated *S. pneumoniae* is used to maximize the exposure of species-common sub capsular antigens. Killing the cells with 70% (vol/vol) ethanol at 4°C produces the most protective preparation than traditional methods of inactivation such as heat, formalin, or UV radiation (Lu, Yadav et al. 2010). Recently, it has been shown however that killing the cells with chloroform, trichloroethylene, or beta-propiolactone without postkilling wash procedure gave more immunogenic preparations than ethanol, expanding the range of agents that can be used to inactivate *S. pneumoniae* whole cells (Lu, Yadav et al. 2010). Intranasal application (thought to be effective for inducing both systemic and mucosal immunity) of ethanol-killed whole cells with cholera toxin as a mucosal adjuvant reduced nasopharyngeal colonisation, middle ear infection and fatal pneumonia caused by encapsulated pneumococci of all tested serotypes in mice (Malley, Morse et al. 2004).

Nontoxic single and double mutants of *Escherichia coli* heat-labile toxin however have been tested as adjuvants and were shown to be as effective as mucosal adjuvants and with these adjuvants protection against colonisation could be induced by the sublingual and buccal routes (Lu, Yadav et al. 2010). Thus, an immunogenic whole-cell antigen can be generated and given in a variety of ways. Research has shown that WCV engages TLR2 and TLR4 in preventing colonisation (Malley, Srivastava et al. 2006). The vaccine also induces serum antibodies in mice when administered prior to challenge (Malley, Lipsitch et al. 2001). Induction of reduced colonisation is CD4+ T cell-dependent and interleukin 17A (IL-17A) mediated and can be antibody independent (Malley, Trzcinski et al. 2005; Lu, Gross et al. 2008). Further in-depth research to demonstrate safety and efficacy in humans will be required in the development of WCV.

1.13.4 Pneumococcal Protein Vaccines

Although capsular polysaccharide-protein conjugate vaccines have been shown to be effective in reducing invasive pneumococcal disease, they are less effective in preventing mucosal disease (20-39% efficacy compared to 77-85% efficacy for systemic disease)((Cutts, Zaman et al. 2005; Qasi S 2007) and it is unclear whether their effects on carriage due to herd immunity are sufficiently long-lived for a setting such as sub-Saharan Africa where pneumococcal carriage rates are very high. Furthermore, their usefulness is limited by restricted capsular serotype distribution, serotype replacement, multiple –dose regimens and high cost. Even the newer generation of conjugates vaccines will partly cover serotypes in developing countries. For instance, a 9-valent conjugate vaccine, would cover 66% of invasive isolates from children and 55% from adults in Malawi (Gordon, Kanyanda et al. 2003) while the 10-valent and 13-valent vaccines would could cover 50% and 60% of invasive isolates in Malawi, respectively (Everett, D, MLW, unpublished data). Several pneumococcal proteins have been identified as potential vaccines which could circumvent some of these drawbacks identified with PCVs and would be synergistic to the capsular polysaccharide approach (Tai SS et al. 2006). Candidate proteins under investigation include pneumolysin and its non-toxic derivatives, choline-binding proteins such as PspA and CbpA, metal-binding proteins, such as PsaA and PiaA and neuraminidase NaA (Table 1.7) and some of these proteins not only contribute to pneumococcal virulence but are produced by virtually by all clinical isolates.

Preliminary studies in mice showed that immunisation of these proteins can protect against infection with multiple serotypes of *S. pneumoniae* and /or prevent nasopharyngeal carriage (Briles, Hollingshead et al. 2000). Immunization with certain combinations of pneumococcal proteins such as a combination of Ply, PspA and CbpA have been shown to provides additive or even synergistic protection (Ogunniyi, LeMessurier et al. 2007). The use of protein-based vaccines raises the possibility of mucosal vaccination which may be a more effective way to protect against mucosal carriage than parenteral immunisation (Jambo, Sepako et al. 2010) and also to induce herd immunity. Protein–based vaccine may also be easier and cheaper to manufacture than polysaccharide vaccines. Additionally, they may not require refrigerated storage and multiple-dose regimens, and hence may be cheaper and easier to administer in resource-poor countries.

Table 1.7 | Mouse models of mucosal immunisation with pneumococcal protein antigens[a] (Jambo, Sepako et al. 2010)

Antigen or vaccine	Route	Immunogenicity	Correlates of protection	Protection against:	Refs
PspA	Intranasal	Mucosal and systemic	Antibodies in serum and saliva	Colonisation, pneumonia, sepsis	(Wu, Nahm et al. 1997; Yamamoto, McDaniel et al. 1997; Campos, Darrieux et al. 2008)
PspA/ attenuated Salmonella	Oral	Mucosal and systemic	Antibodies in serum and vaginal fluids	Pneumonia, bacteraemia	(Nayak, Tinge et al. 1998; Kang, Srinivasan et al. 2002; Park, Ko et al. 2008; Li, Wang et al. 2009; Xin, Li et al. 2009)
PsaA	Oral	Mucosal and systemic	Antibodies in serum, BAL and intestinal fluid	Pneumonia, bacteraemia	(Seo, Seong et al. 2002; Areas, Oliveira et al. 2004; Pimenta, Miyaji et al. 2006)
PsaA/lactic acid bacteria	Intranasal	Mucosal and systemic	Antibodies in serum, saliva, nasal and bronchial washes	Colonisation	(Oliveira, Areas et al. 2006)
PotD	Intranasal	Mucosal and systemic	Antibodies in serum and saliva	Colonisation, pneumonia, bacteraemia	(Shah, Briles et al. 2009)
PsaA and PspA	Intranasal	Mucosal and systemic	Antibodies in serum and saliva	Colonisation	(Briles, Ades et al. 2000)
PspA and PspC	Intranasal	Mucosal and systemic	Antibodies in serum, vaginal washes, and BAL; cytokine responses in BAL, lung and splenic samples	Pneumonia, bacteraemia	(Xin, Wanda et al. 2008; Ferreira, Darrieux et al. 2009)
PsaA, PdT and CWPS	Intranasal	Systemic	Antibodies in serum; T cell response in whole blood	Colonisation, pneumonia	(Lu, Gross et al. 2008; Arevalo, Xu et al. 2009)
GEM with PpmA, SlrA and IgA1p	Intranasal	Systemic	Antibodies in serum	Pneumonia	(Audouy, van Roosmalen et al. 2006)
PCV	Intranasal	Mucosal and systemic	Antibodies in serum and nasal washes	Colonisation, otitis media	(Jakobsen, Saeland et al. 1999; Lynch, Briles et al. 2003; Sabirov and Metzger 2006; Sabirov and Metzger 2008)

[a]Abbreviations: PspA, pneumococcal surface protein A; PsaA, 'pneumococcal surface adhesion A' protein; BAL, bronchoalveolar lavage; PotD, polyamine transport protein D; PspC, pneumococcal surface protein C; PdT, pneumolysin nontoxic derivative; CWPS, cell wall polysaccharide; GEM, Gram-positive enhancer matrix; PCV, pneumococcal conjugate vaccine.

1.14 Current and new approaches for evaluating T cell Immunity

T lymphocytes play an important role in the regulation of immune responses and mediate many of the effector mechanisms of the immune system. Therefore, there has always been a need for assays that can measure accurately the function or activity of T cells in both humans and animal models. This section reviews some of the assays that are commonly used to assess T cell function and describes some new approaches for evaluating T cell immunity.

The available assays measure functions of the responding T cells that occur from relatively early time points (MHC-peptide binding, cytokine production, cytotoxicity) to later time points (proliferation) in the T cell activation process (Figure 1.9). The assays can be categorized according to whether their readout measures a function in bulk cultures (Enzyme Linked Immunoassay (ELISA), Cytometric Bead Array (CBA) and Luminex), or enumerates the responding T cells on a single cell basis (MHC-peptide tetramer staining, enzyme-linked immunospot (ELISpot), Intracellular cytokine staining (ICS), CD107 staining and CFSE assay) (Table 1.8).

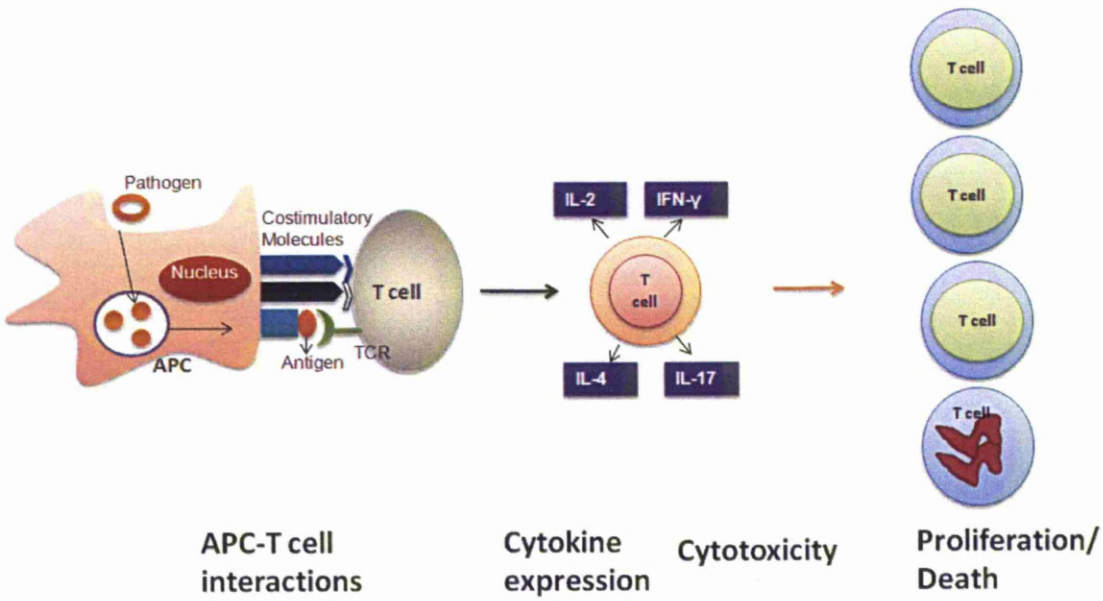


Figure 1.9 | Targets for immune evaluation. Available T cell assays measure functions of the responding T cells that occur from relatively early time points (MHC-peptide binding, cytokine production, cytotoxicity) to later time points (proliferation) in the T cell activation process.

Measurement of proliferative capacity of T cells

Proliferation is a functional feature vital for the T cell control of several diseases (Horton, Frank et al. 2006). Classically, the proliferative capacity of T cells was determined by radioactive thymidine incorporation into the DNA of dividing cells. The assay requires the cells to be stimulated for 3-7 days, before addition of the radiolabelled titrated thymidine (^{3}H-Thy) for 6-8 hours. The total amount of radioactive thymidine incorporated into cells in that time period is then measured, providing a measure of the rate of synthesis of DNA by the entire population of cells (Hickling 1998).

However, the development of non-radioactive flow cytometric assays supplants the need to use radioactivity and provide additional detail about the proliferating antigen-responsive T cells (Bolton and Roederer 2009). An example of a nonradioactive method is carboxyfluorescein succinimidyl ester (CFSE) lymphoproliferation assay. In this case, cells (usually PBMCs) are labelled with CFSE fluorescent dye, incubated with desired antigens and allowed to proliferate for 7-10 days. As the cells proliferate, CFSE is divided equally among daughter cells with each generation becoming half as intense in CFSE staining (Lyons 2000; Wallace, Tario et al. 2008). Halving of cellular fluorescence intensity therefore marks each successive generation in a population of proliferating cells which can be easily identified and enumerated by flow cytometry (Mannering, Morris et al. 2003). CFSE staining can be used in combination with phenotypic and intracellular cytokine staining measurements to provide a detailed view of cell responsiveness.

Cytokine measurement

One of the ways T cells mediate effector mechanisms of the immune system is through the release of cytokines, effector molecules that regulate many of the functions of the cells of innate and adaptive immunity (O'Shea, Ma et al. 2002). One of the mainstays for quantifying cytokines in many laboratories is the enzyme-linked immunosorbent assay (ELISA). The majority of ELISAs are a double antibody method, where a cytokine is sandwiched between two specific antibodies bound to two different, non-competing epitopes of the cytokine (de Jager and Rijkers 2006). One of the antibodies, the capture antibody is immobilised in a solid carrier. The second antibody is labelled either directly or indirectly with a reporter. ELISA is commonly used due to its high specificity and sensitivity.

Additionally, it is relatively cheap, require little instrumentation, is easy to automate and no radioactive ingredients are used. ELISA methods however generally analyse one cytokine at a time and therefore they are not well suited for high throughput multiplex analysis (de Jager and Rijkers 2006) and do not identify the cells producing the cytokines. Studies show that measurement of a single cytokine is often not sufficient to obtain a realistic idea of the complexity of biological interactions that occur at the cellular level and underestimates the magnitude of antigen-specific responses. Due to an increased need for more rapid, sensitive, robust and high throughput methods for getting a full spectrum of cytokines from a single often-limited amounts of sample, fluorescent-labelled bead based assay have emerged similar to ELISA (Elshal and McCoy 2006).

Bead array assays may be carried out on either multi-use flow cytometry or on more specialised platforms such as Luminex 200 system. The two most widely used fluorescent bead based assays are the Cytometric bead array (BD Biosciences) and the xMAP technology (Luminex). In both assays, the bead set is coated with capture antibody specific for a particular cytokine. When a sample is combined with the antibody-coupled beads, the cytokine of interest is captured through a biochemical binding reaction. The amount of cytokine bound to each bead population is shown by staining intensity of the detection reagent (Bolton and Roederer 2009).

Multiple bead array methods (MBA) allow simultaneous measurements of many cytokines, cover a greater dynamic range (minimising the need to carried out multiple dilutions) and use far less specimen material. However, they do not identify the cells that elicit cytokine(s), nor the patterns of co-expression by cytokines at the level of an individual cell (Bolton and Roederer 2009).

Another commonly used method for assaying cytokine production is the intracellular cytokine staining (ICS) method. ICS is a method that permits simultaneous measurements of multiple cytokine production produced at the level of an individual cell. In the ICS method, cells are stimulated with desired antigens and in the presence of cytokine secretion inhibitor (Brefeldin A [BFA] or Monensin) for at least some of the stimulation period. The cells are then fixed and permeabilised to be stained intracellularly with anti-cytokine fluorescent antibodies. ICS offers several advantages: it allows identification of cells that elicit cytokines other than IFN-γ (a cytokine commonly detected by ELISpot assay) expanding the range of antigen-responsive T cells that can be detected.

It also allows simultaneous determination of the phenotype of responding T cells, reducing the need to do multiple assays on often-limited amounts of sample.

Enzyme-linked immunospot (ELISpot)

The enzyme-linked immunospot (ELISpot) assay is an adaptation of the ELISA and is widely used for monitoring T cell immune responses and validating new T cell epitopes (Mashishi and Gray 2002; Karlsson, Martin et al. 2003). PBMCs are plated on a filter-bottom 96-well plate coated with anti-cytokine antibody. The cells are incubated with antigens for 18- 24 hours to allow cytokine secretion and capture on the plate. Cells are washed off and detector antibody is added, followed by enzyme substrate. Cytokine-producing cells are identified as spots of secreted cytokine. This assay is 200 times more sensitive than ELISA, relatively inexpensive and non-radioactive (Hickling 1998). However, the assay only quantifies a single function and as such may underestimate the number of antigen-specific T cells. Additionally, it provides no information on the cytokine-secreting cells (Bolton and Roederer 2009).

Helper function

CD4+ T cells provide essential helper function for antigen-presenting cells (e.g. dendritic cells, monocytes and B cells). This function is mediated partly by CD154 (on the CD4 T cell) via binding to CD40 on the antigen-presenting cells (Miga, Masters et al. 2000; Straw, MacDonald et al. 2003). CD154 is nearly globally expressed by T cells upon activation and therefore the measurement of CD154 can serve as an global indicator of the capacity of CD4+ T cells to provide helper function (CD154-mediated help) and as a surrogate marker for antigen-responsive T cells. CD154 synthesis is assessed by flow cytometry and can be coupled to multiplexed measurements of cytokine expression, providing more details on the function of antigen-specific CD4+ T cells. It has been shown that CD154 marks a wide variety of antigen –specific CD4 T cells, including nearly all cells that express IFN-γ, IL-2 and/or TNF-α (Chattopadhyay, Yu et al. 2005). Since surface staining for CD154 is not lethal, it provides a means to isolate and purify viable antigen-responsive CD4+ T cells for further analysis.

Table 1.8 | Assays for evaluating T cell immunity

Function	Bulk Assay	Single-cell Assay
Proliferation		3^{H} –thymidine incorporation Advantages • relatively easy to do Disadvantages • Use of isotypes • Identify proliferating cells unknown • Functional properties unknown CFSE labelling Advantage • Relatively easy to do • Allows identification of cells • No use of isotypes Disadvantages • Identifies proliferating cells only
Cytokine	ELISA Multiples bead assay - Cytometric Bead Array - Luminex-xMAP Advantages • Relatively simply & robust • Suitable for high throughput screening Disadvantages • Identity of cytokine producing cells not always known • Patterns of coexpression of cytokines from individual cells unknown • Can be very expensive	ELISpot Advantages • Sensitive, reliable and relatively inexpensive • Suitable for high through put testing Disadvantages • Identity of cytokine producing cells not always known • May underestimated the number of antigen-specific cells Intracellular Cytokine staining Advantages • Sensitive, reliable and relatively inexpensive • Suitable for high throughput testing
T helper function		CD154 expression Advantages • Ease to perform • Compatible with ICS • Allow purification of viable cells
T cell specificity		MHC-peptide tetramer staining Advantages • Ease to perform • Identifies all epitope specific T cells • Simple detection by flow cytometry • Can be performed ex vivo • Depends on frequencies Disadvantages • Number of available MHC class I peptide complexes is limited • Must know HLA background of study subjects • For defined epitopes only
Cytotoxicity	^{51}Chromium release assay Disadvantages • Use of isotopes • Effector cells identity unknown • Relatively insensitive • Expansion of T cells required	CD107a expression Advantages • Ease to perform • Compatible with other assays e.g. tetramer binding Disadvantages • May overestimate cytotoxicity

MHC multimer staining

Antigen-responsive T cells can also be assessed by flow cytometric detection of cells stained with fluorescently labelled peptide-MHCs (tetramers), a technique known as 'MHC tetramer staining' (Altman, Moss et al. 1996). The technique allows detection of antigen-specific T cells based on the binding specificity of their cell surface receptors for specific MHC-peptide complexes. The main advantage of this assay is its ability to identify rare populations of antigen-specific T cells without *in vitro* activation. This is important for assessing cellular properties that correlate with antiviral and tumour immunity, for example memory phenotype (Wherry, Teichgraber et al. 2003; Klebanoff, Gattinoni et al. 2005; Bolton and Roederer 2009). It can also identify T-cell epitopes important for controlling disease (Palmowski, Salio et al. 2002). Flow cytometric analysis of MHC tetramer allows phenotypic analysis and isolation of antigen-responsive cells for further analysis (Vollers and Stern 2008). The major disadvantage of tetramer staining is that the tetramer can only be used on a subject with matching haplotype but the number of available MHC class I peptide complexes is limited and the development of CD4 T cell tetramers has been impeded by the folding constraints for recombinant class II MHC proteins (Vollers and Stern 2008; Bolton and Roederer 2009). Furthermore, peptides to be loaded onto the MHC must be determined for each disease.

CTL cytotoxicity assays

Cytotoxic CD8 T cells (and the rarer cytotoxic CD4 T cells) play a critical role in the control of intracellular pathogens and malignant tumour cells. The gold standard assay for determining the number of antigen-specific killer cells is the chromium (^{51} Cr) –release assay (Hickling 1998). In this assay, the 'target cells' are radiolabelled with sodium chromate ($Na_2{}^{51}CrO_4$) and then incubated with the test effector cell population for 4-6 hours. The amount of ^{51} Cr released into the supernatant is then quantified, to provide a measure of target-cell lysis. However, there are several nonradioactive flow cytometric assays that are often used to detect functional antigen-specific CTLs and one such assay is the flow cytometric assay for degranulation (Betts, Brenchley et al. 2003).

The granule-associated integral membrane proteins CD107a and CD107b are proteins found in cytotoxic granules of CTL. Upon degranulation, CD107a and CD107b are transiently transported to the cell surface.Using labelled antibodies to CD107a and CD107b during a short-term stimulation, the exocytosis of CD107 is captured on degranulating cells.

CD107a staining correlates well with ^{51}Cr-release (Rubio, Stuge et al. 2003). Although degranulation is a requirement for cytotoxicity, not all degranulated cells are cytotoxic (Bolton and Roederer 2009). C107a/b staining has been found to be useful for determining the effectiveness of therapeutic vaccines (Betts, Brenchley et al. 2003; Rubio, Stuge et al. 2003). It can also be used in combination with other assays such as tetramer binding.

1.15 Study Rationale

S. pneumoniae is an important HIV-related pathogen in sub-Saharan Africa. HIV-infected adults are 20 to 100 times more likely to suffer invasive pneumococcal disease (IPD) than age-matched HIV negative persons and are at increased risk of pneumococcal infection at all stages of HIV-1 infection. In Malawi, pneumococcal bloodstream infection is one of the common reasons for hospital admission amongst HIV-infected adults. Though some restoration of immunity has been reported following antiretroviral therapy (ART) coupled with reduction in HIV-1 related opportunistic infections and a reduction in the incidence and magnitude of IPD, HIV positive persons are still at a greater risk of IPD compared to uninfected individuals.

Questions remain as to how pneumococcal immunity changes with HIV-infection, immune reconstitution and vaccination. We hypothesize that immunity to *S. pneumoniae* (which is thought to rely on T and B cell memory) is considerably compromised in HIV-infected persons and is only partially restored by antiretroviral therapy. A better understanding of the influence of HIV infection on pneumococcal immunity in the adult population can inform design and implementation of both antiretroviral therapy (ART) and protective vaccines.

This thesis proposes to:

1- Investigate the impact of HIV on naturally-acquired cellular to pneumococcal antigens. This will provide an in-depth mechanistic understanding of the impact of HIV on pneumococcal immunity.

2- Investigate the impact of ART on naturally-acquired immunity to pneumococcal antigens, mapping out the kinetics and the degree of immune reconstitution. The kinetics and the degree of recovery of pneumococcal immunity with antiretroviral therapy will shed light as to what is the right time to immunise with existing and novel protein-based pneumococcal vaccines.

3- Provide preliminary data on the relationship between naturally-acquired immunity and carriage of *Streptococcus pneumoniae* in HIV-infected adults. The study will provide important insights into how *S. pneumoniae* carriage changes during immune reconstitution and will inform the likelihood of vaccine being able to achieve herd immunity through the eradication of pneumococcal carriage.

4- Evaluate immune responses elicited by PCV7 vaccination and to compare the differences in immune responses mounted by healthy HIV uninfected adults with HIV-infected adults. The study seeks to determine whether HIV-1–infected patients are capable of mounting significant and sustained immune responses to the pneumococcal antigens and carrier protein after vaccination.

CHAPTER 2 SUBJECTS, MATERIALS AND METHODS

2.1 Research Setting

2.1.1 Malawi

Malawi is a landlocked country in southeast Africa with a population of 13 571 000. Malawi is one of the poorest countries in the world with a *per capita* Gross National Income of US$160(~690 PPP international $) (Figure 2.1)(WorldVision 2008; WHO 2008a). The overall per capita expenditure on health is only US$14 (Intl $, 2005:~ 64) and private financing accounts for 59% of the total expenditure. HIV seroprevalence in Malawi is around 14% in adults (WHO 2008b), rising to 18-28% in Blantyre antenatal clinics. Highly active anti-retroviral therapy (HAART) became available in 2003 and as at the end of December 2009, a total of 198 846 (73%) individuals were alive on therapy (*Malawi ART Programme Report for Quarter 4 2009).*

HIV/AIDS and lower respiratory infections were the top two causes of death in all ages in Malawi in 2002 (Figure 2.2a)(WHO 2006), both remain prominent causes of mortality. Additionally, *S. pneumoniae* (17.2%) and *S. typhimurium* (32.4%) accounted for nearly 50% of all cases of bacteraemia in Malawian adults in 2008 (Figure 2.2b) (*Malawi-Liverpool Wellcome Trust Clinical Research Programme: Core Laboratory).*

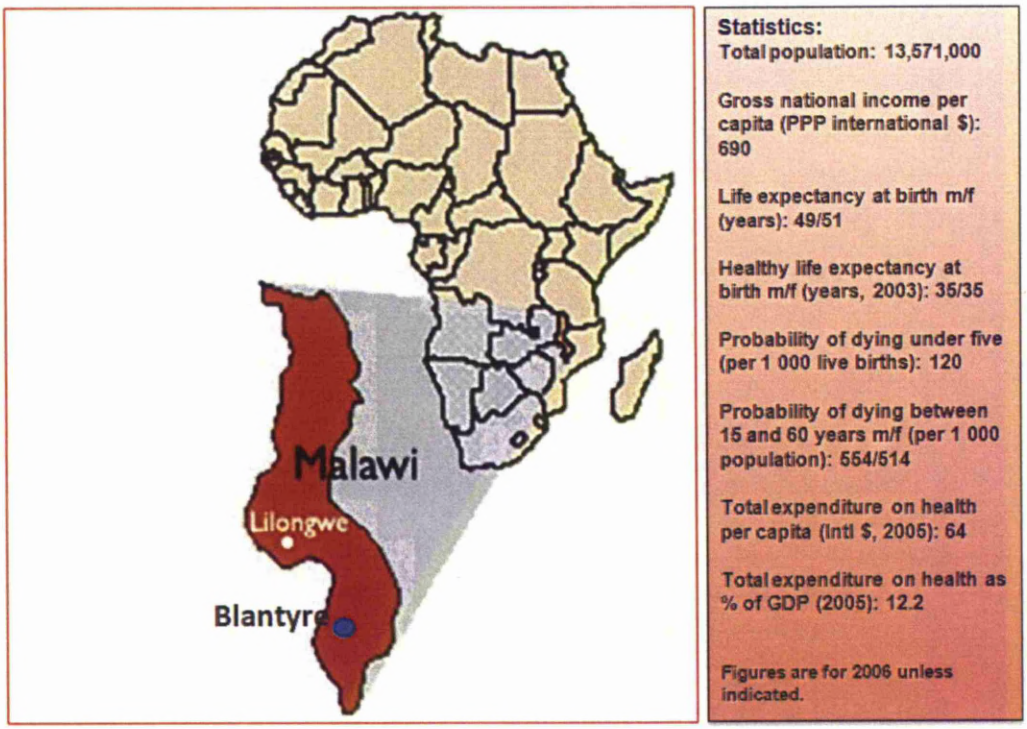
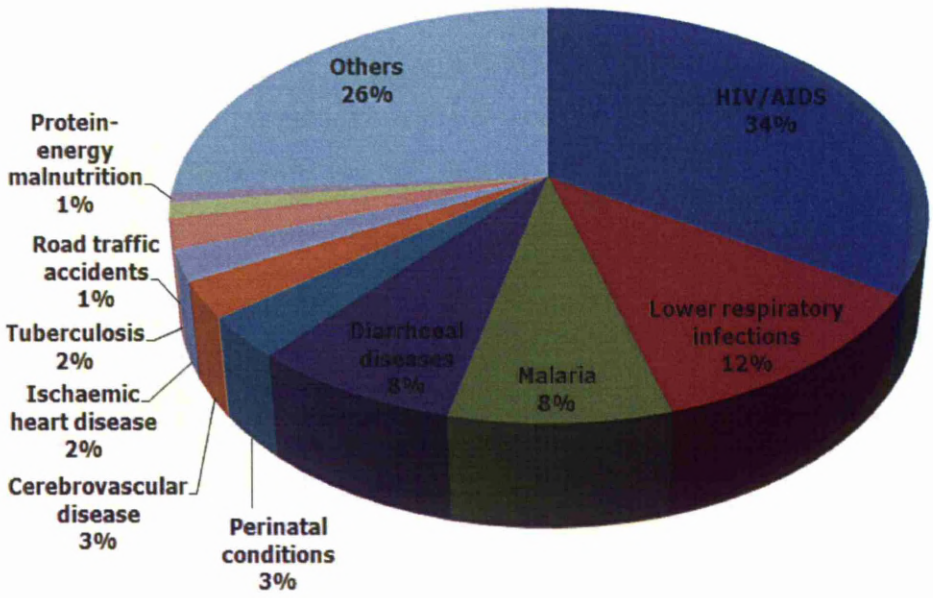


Figure 2.1 | Malawi: Geographic location and country statistics (WorldVision 2008; WHO 2008a)

a



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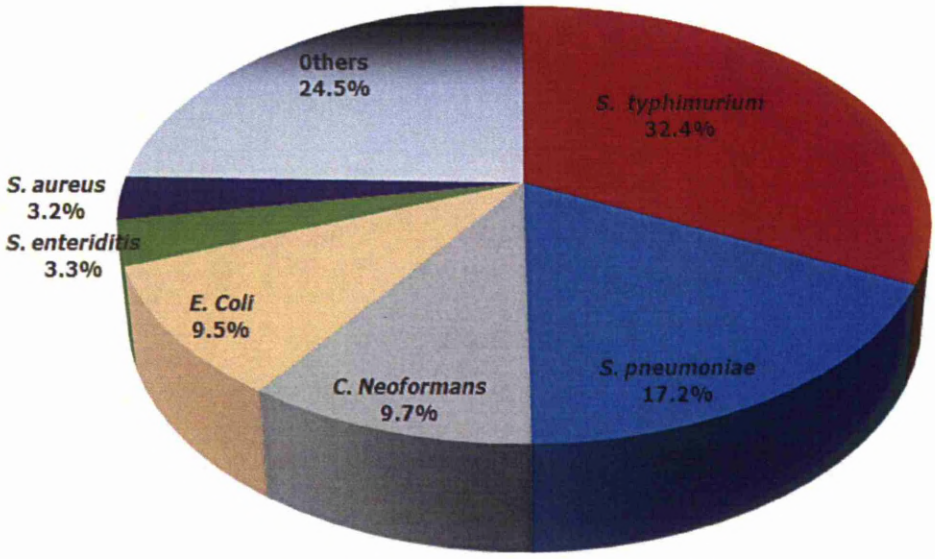


Figure 2.2 | Causes of death and bacteraemia in Malawi. **a|**Top ten causes of death in all ages in Malawi in 2002 (WHO 2006) **b|**Causes of bacteraemia in Malawian adults in 2008 (*Malawi-Liverpool Wellcome Trust Clinical Research Programme: Core laboratory*).

2.1.2 The Malawi-Liverpool Wellcome Trust (MLW) Clinical Research Programme

The Malawi-Liverpool-Wellcome Trust (MLW) Clinical Research Programme (www.mlw.medcol.mw) is based in Blantyre, Malawi, at its Wellcome Trust-funded research laboratories. The laboratories were opened on 22 January 1999 and are located on the grounds of the Queen Elisabeth Central Hospital (QECH). The Programme is a research affiliate of the College of Medicine, University of Malawi and investigates health problems relevant to Malawi and the region. The research activities are focused under five large multidisciplinary research themes to develop multiple synergistic projects namely, malaria and brain diseases, therapeutics in the tropics, severe bacterial infection, mucosal and vaccine immunity and health in population (http://www.mlw.medcol.mw/research.html). The Programme has strong scientific and administrative links with the University of Liverpool and the Liverpool School of Tropical Medicine (LSTM), UK.

2.2 Study population

2.2.1 Cross-Sectional Pneumococcal immunity-HIV infection Study in Malawian Adults (Chapter 4)

The aim of the study was to investigate the impact of HIV on naturally-acquired pneumococcal immunity. The study was conducted in compliance with relevant guidelines and institutional practices of LSTM, UK and the Malawi-Liverpool Wellcome Trust Clinical Research Programme, Blantyre, Malawi and was approved by the College of Medicine (University of Malawi) and LSTM research ethics committees (Protocols: P.03/08/626 and 08.41 respectively). Recruitment followed informed consent.

Sample size

It was anticipated that >70% of control adults will have B cell and T cell immunity to the pneumococcus. At the time the proposal was written there were no data on cellular immune responses to the pneumococcus in HIV infected individuals, on which to base a sample size calculation. However, with 100 HIV-1 positive patients and 40 HIV-1 healthy volunteers, there was an 80% power to detect a difference in means of 0.53S, assuming responses are normally distributed with equal variability, using a two-sample t-test and a 5% significance level (S= standard deviation). At the end of the study, we had recruited 53 HIV-infected individuals and 31 HIV-uninfected persons.

2.2.1a HIV-seronegative adults: Participants for the study were drawn from healthy uninfected adults visiting the voluntary counselling & testing (VCT) clinic, Queen Elisabeth Central Hospital, Blantyre, Malawi or recruited by advertisement in the hospital grounds. Persons visiting the VCT clinics for HIV testing are tested with 2 complimentary rapid tests; Unigold^{TM} (Trinity biotech, Ireland) and determine (Abbot, Japan). Samples were collected from consenting adults who met the prescribed inclusion and exclusion criteria (see criteria below). To ensure that individuals in the non-HIV control group were HIV seronegative the samples were retested at the MLW Clinical diagnostic laboratory.

HIV testing was done using 2 complimentary rapid tests; Unigold^{TM} (Trinity biotech, Ireland) and determine (Abbot, Japan) according to manufacturers' instructions. In the case of disparate results, a 3rd test was done using the Bioline test kit (Standard Diagnostics Inc, Korea).

Inclusion criteria for HIV-seronegative adults

- Age ≥18 years and <55years
- HIV negative
- In good health at the time of entry into the study as determined by medical history, physical examination and clinical judgment of the investigator
- Not known to be pregnant

Exclusion criteria for HIV-seronegative adults

- HIV infection by serological test
- Febrile (≥38.0°C axillary) or unwell
- Patients with previous or current TB
- Other immunocompromising illness e.g. diabetes, cancer data
- Known or possible pregnancy
- Immunosuppressant drugs e.g. steroids
- Any condition which, in the opinion of the investigator, might interfere with the evaluation of the study objectives
- Severe anaemia HB <8 g/dl

2.2.1b HIV infected adults: Participants were recruited from healthy infected adults (WHO stage 1 [Appendix I]) attending anti-retroviral (ARV) out-patient clinics or visiting the VCT clinic of Queen Elisabeth Central Hospital, Blantyre, Malawi. Samples were collected from HIV-positive consenting adults who meet the prescribed inclusion and exclusion criteria (see below).

Inclusion criteria for HIV-1 infected cases

- HIV infection by serological test and clinical features of WHO stage 1 (asymptomatic or persistent generalized lymphadenopathy)
- Age ≥18 years and <55year
- In good health at the time of entry into the study as determined by medical history, physical examination and clinical judgment of the investigator
- Not known to be pregnant

Exclusion criteria for HIV-1 infected cases

- Febrile (T ≥38.0°C axillary) or unwell
- Unexplained weight loss, recurrent respiratory infections, or Herpes zoster
- Patients with previous or current TB or cryptococcal disease
- Patients who are taking antiretroviral treatment.
- Other immunocompromising illness e.g. diabetes, cancer data
- Known or possible pregnancy
- Immunosuppressant drugs e.g. steroids
- Any condition which, in the opinion of the investigator, might interfere with the evaluation of the study objectives
- Severe anaemia HB <8 g/dl

2.2.2 Longitudinal Anti-Retroviral Therapy Study in Malawian Adults (Chapter 5)

The aim of the study was to investigate the impact of antiretroviral therapy on naturally-acquired immunity to pneumococcal protein antigens, mapping out the kinetics and the degree of immune reconstitution.

The study was conducted in compliance with relevant guidelines and institutional practices of LSTM, UK and the Malawi-Liverpool Wellcome Trust Clinical Research Programme, Blantyre and was approved by the College of Medicine (University of Malawi) and LSTM research ethics committees (Protocols: P.07/09/801 and 09.71 respectively). Recruitment followed informed consent.

Sample size

Based on our initial work on cellular immune responses to the pneumococcus in HIV infected adults (Chapter 4 of this thesis), we proposed that with 52 HIV-1 positive patients, there will be 85% power to detect a difference in means of 0.300, assuming responses are normally distributed with equal standard deviation of 0.5 using a two-sample t-test and 5% significance level. Allowing for subject tolerance of the sample correction procedures, we anticipated that we will recruit 60 HIV-1 positive adults 30 HIV uninfected healthy volunteers. At the time of writing this thesis, we had recruited 48 HIV-positive patients initiating ART and 14 healthy HIV-uninfected individuals.

2.2.2a HIV-seronegative adults: Participants for the study were drawn from healthy uninfected adults visiting the voluntary counselling & testing (VCT) Clinic, Queen Elisabeth Central Hospital, Blantyre, Malawi or recruited by advertisement in the hospital grounds. Samples (venous blood and nasopharyngeal swabs) were collected from consenting adults who met the prescribed inclusion and exclusion criteria (see criteria below).

Inclusion criteria for HIV-uninfected volunteers

- Age ≥18 years and <55years
- HIV negative
- In good health at the time of entry into the study as determined by medical history, physical examination and clinical judgment of the investigator
- Not known to be pregnant

Exclusion criteria for HIV-uninfected volunteers

- HIV infection by serological test
- Febrile (≥38.0°C axillary) or unwell
- Patients with previous or current TB
- Other immunocompromising illness e.g. diabetes, cancer data

- Known or possible pregnancy
- Immunosuppressant drugs e.g. steroids
- Any condition which, in the opinion of the investigator, might interfere with the evaluation of the study objectives
- Severe anaemia HB <8 g/dl

2.2.2b HIV infected adults: Participants were recruited from HIV-infected adults commencing on anti-retroviral therapy (ART) at the anti-retroviral out-patient clinics of Queen Elisabeth Central Hospital, Blantyre. Samples (venous blood and nasopharyngeal swabs) were collected from consenting adults who met the prescribed inclusion and exclusion criteria (see criteria below). Samples were collected at the time of enrolment and the participants were followed up for a period of 12 months during which samples were collected at 3, 6 and 12 months.

Inclusion criteria for HIV-1 infected cases

- HIV infection by serological test and eligible for ART therapy (clinical stage III or IV, or CD4 count <250)
- Age ≥18 years and <55years
- In good health at the time of entry into the study as determined by medical history, physical examination and clinical judgment of the investigator
- Not known to be pregnant

Exclusion criteria for HIV-1 infected cases

- Febrile (T ≥38.0°C axillary) or unwell
- Unexplained weight loss, recurrent respiratory infections, or Herpes zoster
- TB or cryptococcal disease in the past six months
- Patients already on antiretroviral treatment.
- Other immunocompromising illness e.g. diabetes, cancer data
- Known or possible pregnancy
- Immunosuppressant drugs e.g. steroids
- Any condition which, in the opinion of the investigator, might interfere with the evaluation of the study objectives
- Severe anaemia HB <8 g/dl

2.2.3 Longitudinal Pneumococcal Conjugate Vaccine Study in Malawian Adults (Chapter 6)

The aim of the study was to investigate immunological memory mounted in response to vaccine antigens in HIV-infected Malawian adults well established on ART. The study was considered and approved by the institutional review boards of the College of Medicine of University of Malawi (College of Medicine Research and Ethics Committee- COMREC) and Liverpool School of Tropical Medicine , UK (Protocols: P.05/08.665 and 08.64 respectively) and recruitment followed informed consent.

Sample size

The sample size was constrained by access to past PCV7 vaccine trial participants (placebo arm). The proposed numbers (40 HIV-infected and 15 HIV-uninfected persons) were a pragmatic number based on the follow-up rates of the clinical trial (137 HIV-infected and 19 HIV uninfected subjects). With these numbers we anticipate to detect a moderate difference in immune response between 2 groups with 80% power. For example, if responses are normally distributed with standard deviation (s), then differences of 0.63s will be detectable for comparison of the groups(s = standard deviation). At the end of the study, we had recruited 32 HIV-infected individuals and 15 HIV-uninfected individuals into the study.

2.2.3a HIV-seronegative and HIV infected adults: Participants for the study were drawn from adults who were previously enrolled (as controls) in a double-blinded, randomized, placebo-controlled clinical efficacy trial of a 7-valent conjugate pneumococcal vaccine (PCV7) (French, Gordon et al. 2010). The recruitment was done at the Pneumovac clinic, Queen Elisabeth Central Hospital, Blantyre, Malawi. Samples (venous blood and nasopharyngeal swabs) were collected from consenting adults who met the prescribed inclusion and exclusion criteria (see criteria below). Samples were collected at the time of enrolment and the participants were followed-up for a period of six (6) months during which samples were collected at 8 weeks and 24 weeks post enrolment (Figure 2.3).

Inclusion criteria for study participants

- Age ≥18 years <55years
- In good health at the time of entry into the study as determined by medical history, physical examination and clinical judgment of the investigator
- Not known to be pregnant
- Never received PCV-7 vaccine (but previously in PCV7 trial placebo group)
- Willing to have an HIV test if not known to be HIV positive (HIV-seronegative group)

Exclusion criteria for study participants

- Severe anaemia Hb <8 g/dl
- Febrile (≥38.0°C axillary) or unwell
- Patients with known TB
- Other immunocompromising illness (apart from HIV) e.g. diabetes, cancer data
- Pregnant
- Immunosuppressant drugs e.g. steroids
- Any condition which, in the opinion of the investigator, might interfere with the evaluation of the study objectives.

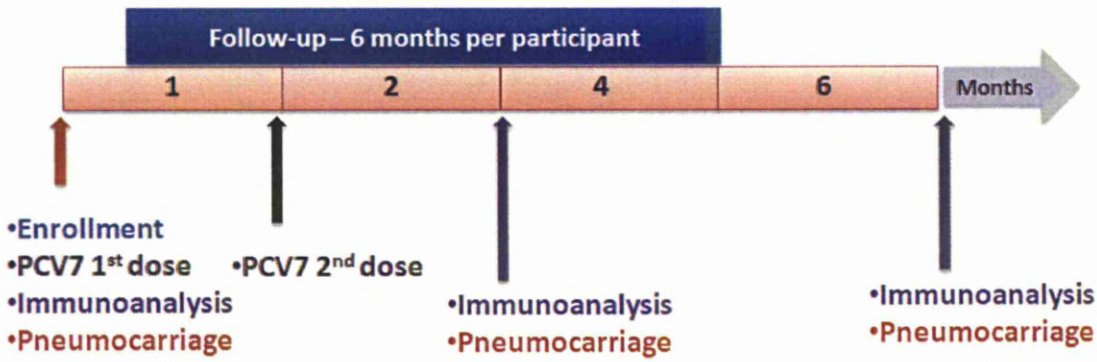


Figure 2.3 | Vaccination Schedule and follow-up. Two doses of the 7-valent conjugate pneumococcal vaccine (PCV7) were given 4 weeks apart, after which patients were requested to come at 4 weeks and 20 weeks. Samples of venous blood and nasopharyngeal swabs were collected at these visits to assess vaccine-induced immunity and pneumococcal carriage.

2.3 Sample Collection and Processing

2.3.1 Venous blood

Approximately 45 ml of blood was drawn by venipuncture into preservative free, sodium heparin vacutainer tubes (BD, UK) for PBMCs isolation and 5 ml into EDTA vacutainer tubes (BD) for diagnostic tests: malaria, differential haematology, CD4 T cell count and plasma for viral load quantification (performed by the MLW Core Laboratory).

2.3.2 Peripheral Blood Mononuclear Cells Isolation

Fresh PBMCs were isolated from whole blood by density-gradient centrifugation. Briefly, whole blood was diluted in HBSS (Gibco) (1:2[blood:HBSS]) and carefully layered on top of lymphoprep (Axis-Shield, UK) at a ratio of 1:2.5 (lymphoprep:diluted blood) in a 50 ml polypropylene conical tube and spun at 700 x g for 25 minutes at room temperature with the centrifuge break removed. After centrifugation, the erythrocytes and granulocytes settled at the bottom of the tube, while the mononuclear cells remained at the plasma-ficoll interface appearing as an opaque layer. The mononuclear cells were carefully harvested into a clean 50 ml polypropylene tube. The cells were washed twice with HBSS by spinning for 15 minutes at 650 x g. The cells were resuspended in RPMI medium (Gibco) containing 20 mM HEPES, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin (Sigma) and counted on a Bright line Haemocytometer (Hausser Scientific) using white blood cell (WBC) counting media [18ml dH_20, 380µl 1M acetic acid and 180ul 0.4% weight/volume trypan blue (dye exclusion stain, Sigma)]. Trypan blue was used to assess cell viability. Cells with intact membranes exclude the dye while cells without intact membranes (dead cells) do not and hence their cytoplasm stain blue (Strober 2001).

2.3.3 T Lymphocytes Enumeration and Differential Haematology

Absolute counts of T lymphocytes were enumerated using BD FACSCount™ reagent kit (CD4/CD8/CD3) (BD, UK) on a BD FACSCount™ instrument (BD Biosciences). Differential haematology was done using COULTER® HmX Haematology Analyser (Beckman Coulter).

2.3.4 Nasopharyngeal Swabs

Nasopharyngeal swabs were obtained using calcium alginate fibre tipped ultrafine alum applicate swab (MWE Medical Care, UK) (O'Brien and Nohynek 2003). Briefly, the swab was held by the shaft, and the tip of the swab inserted into the anterior nares. The medial wall was followed till a resistance was felt and then the swab was rotated through 180 on its axis to ensure good mucosal contact. The swab was carefully removed from the nose and immediately placed into a vial containing 3ml of skim milk-tryptone-glucose-glycerol (STGG) transport medium (avoiding contamination) and the handle cut. The swabs were transported to MLW Clinical diagnostic laboratory in a cool box within 2 hours of collection where the swabs were kept at room temperature overnight before processing.

2.3.5 Detection of upper respiratory carriage of *Streptococcus pneumoniae*

Pneumococcal carriage was evaluated using the World Health Organisation standard protocol for detecting nasopharyngeal pneumococcal colonisation (O'Brien and Nohynek 2003). The nasopharyngeal swab specimen in STGG media was mixed thoroughly and plated onto a gentamicin (2.5µg/ml) sheep blood agar (BA) for the isolation of *S. pneumoniae.* The plate was incubated at 37°C, 5% CO_2 overnight and pneumococci identified by their morphology (alpha-haemolytic colonies). From the primary plate, two presumptive pneumococcal colonies were picked and streaked out on one half of a blood agar and an optochin 6-mm size disc placed in the centre of each streak. The plate was incubated at 37°C, 5% CO_2 overnight. Susceptibility to optochin was defined as the diameter of inhibition zone and depended on the disc size. Zones of inhibition greater than 14 mm indicated susceptibility, 7 to 13 mm indicated indeterminate and less than 7 mm as resistant to optochin. Isolates that were susceptible to optochin were considered pneumococci and those that were resistant to optochin were considered to be a species other than pneumococcus (Figure 2.4).

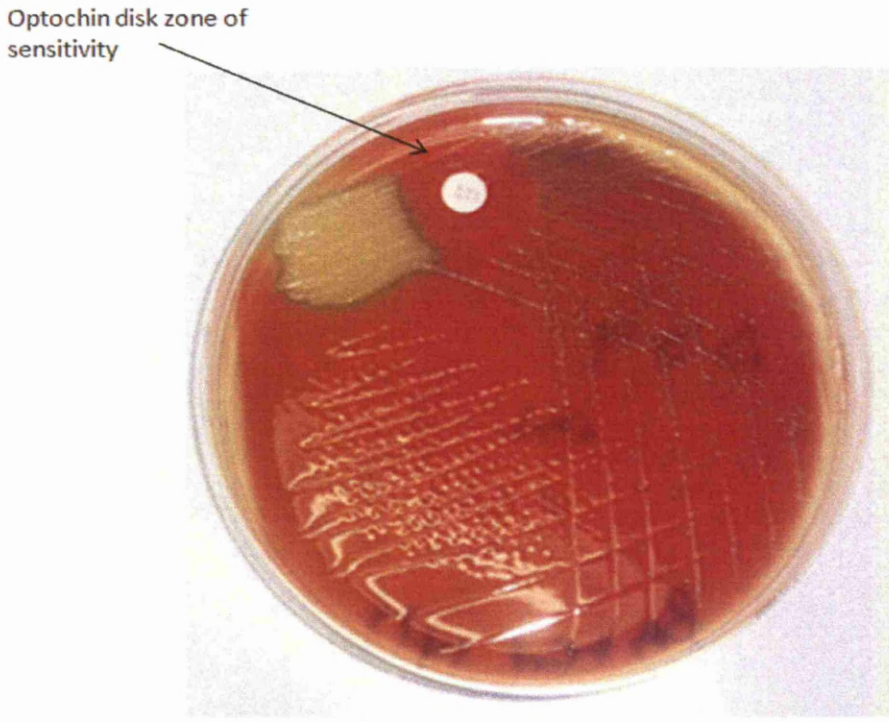


Figure 2.4 | Optochin sensitive pneumococci growing in Gentamicin blood agar (BA) plate - plate after incubation

2.3.6 Diagnosis of Malaria infection

Light microscopy of thick stained blood smears was used to exclude malaria infection (Lewis SM 2005). The slides were prepared at the study clinics in Queen Elisabeth Central Hospital and sent to MLW malaria diagnostic laboratory for staining and examination. To prepare the slides, a drop of blood (6 -12 ul) obtained by puncturing the ball of a finger was placed on a microscope slide and spread to make a 10 mm circle and the filmed was then air dried. The correct thickness of the film is the one through which newsprint is barely visible.

In the laboratory, the blood smears were stained using the Field's stain method. The slides were dipped in acetone for 10 minutes and then in Field's Stain A (Merck, South Africa) for 5 seconds. Next, the slides were gently rinsed in clear tap water for about 5 seconds and stained with Field's Stain B (Associated Chemical Enterprises, South Africa) for 5 seconds and the stain removed by rinsing in clear tap water. The slides were drained vertically and left to dry.

The films were examined under an Olympus Ch30 microscope (Olympus Optical Co. Ltd, Japan) for at least 10 minutes (100 to 200 high power fields) before the slide could be declared negative. In a well stained positive film the parasites are seen as deep red chromatin and pale blue cytoplasm.

2.4 Antigens and Antibodies

2.4.1 Pneumococcal Strains

The study used whole pneumococcal protein antigens prepared from culture supernatants of a standard encapsulated type 2 strain (D39), a pneumolysin deficient mutant encapsulated type 2 (D39) isogenic pneumococci (Ply-) and a genetic toxoid derivative of pneumolysin (PdB).

(kindly provided by Dr James Paton, Adelaide, Australia)(Berry, Ogunniyi et al. 1999). The culture supernatants were used at a final concentration of 8µg/ml and PdB at a final concentration of 4µg/ml.

2.4.1.1 Preparation of Pneumococcal Culture Supernatants

Each pneumococcal strain (standard encapsulated type 2 (D39) and an isogenic pneumolysin-deficient mutant (D39Ply-) was grown on blood agar plates. A pure colony showing an α-haemolytic pneumococcal type growth and optochin (an antibiotic) sensitivity was seen. A few colonies were picked and cultured in Todd-Hewitt broth (Oxoid, UK) supplemented with 0.5% yeast extract (THY) in 5% CO_2 at 37^0C to exponential phase (optical density of 0.4 to 0.5 at 620 nm, approx 1 x 10^8 cfu ml^{-1}). Growth was terminated by centrifugation (300g; 30 min) (Mureithi, Finn et al. 2009). Following centrifugation, the supernatant was collected and initially filtered through a 0.45 µM-pore-size sterile filter (Sigma), and then through a 0.2 µM-pore-size sterile filter (Sigma). The filtered supernatant was concentrated (10-fold) by ultrafiltration using the Vivaspin 20 ml centrifugal concentrator (10 000 MWCO PES) (Sartorius Stedim biotech, UK). Briefly, the culture supernatant was placed in the concentrator chamber and centrifuged, according to manufacturer's instructions. During centrifugation, solutes with low molecular weight as well as solvents pass through the membrane and into the filtrate vial whereas macrosolutes (i.e. concentrated sample) remains in the impermeable concentrate pocket.

2.4.1.2 Determination of Colony Forming Units in pneumococcal cultures

The colony forming units in the pneumococcal cultures were determined using the Miles and Misra method (Heritage J 1996). Briefly, a blood agar plate was divided into six numbered sections (Figure 2.5). Serial dilutions of the pneumococcal culture were prepared as required using sterile PBS. Three drops (each 10 ul) of each dilution were deposited in three different areas of the corresponding section of the blood agar plate (Figure 2.5). The first and second dilutions were not used as they tend to have many colonies with confluence to make viable counts. The samples were left to dry on the plate before incubation overnight in 5% CO_2 at 37°C.

The following day, colonies were counted in each section where more than 20 colonies were present without any confluence and divided by three. The colony forming units per ml were calculated as follows:

i. dilutions made x amount inoculated = " plated dilution"
ii. dilution factor (inverse of plated dilution) x number of colonies = **CFUs/ml** (of the undiluted culture)

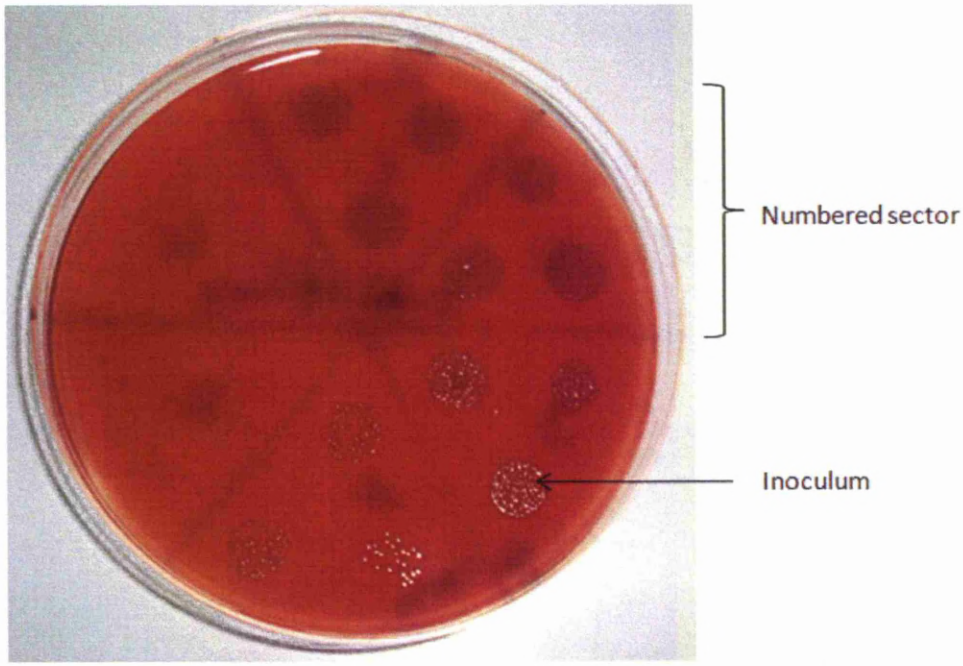


Figure 2.5 | Determination of pneumococcal colony forming units by the Miles & Misra method – plate after incubation

2.4.1.3 Bradford Protein Assay

The protein concentrations of the pneumococcal concentrated culture supernatants (PneumoCCS) were determined using the Bradford protein assay (Bradford 1976; Stoscheck 1990). The assay relies on the binding of the dye Coomassie Blue G-250 to protein. When Coomassie Blue G-250 dye binds to a protein, the absorbance maximum of the dye shifts from 465 nm (the reddish/brown form of the dye) to absorbance maximum of 610 nm (blue form of the dye). The difference between the two forms of the dye is greatest at 595 nm, hence it is the optimal wavelength to measure the blue colour from the Coomassie dye-protein complex.

Five standards of bovine serum albumin (BSA, Sigma-Aldrich) with protein concentrations ranging from 23 to 375 micrograms were used as assay standards. Where necessary the pneumoCCS were diluted with Todd-Hewitt broth plus 0.5% yeast extract (THY) to obtain protein concentrations that fall within the range of the assay standards. Bradford reagent (Sigma) was added to an equal volume of the aliquots of each standard and concentrated culture supernatants. They were then incubated at room temperature for 5 minutes and the Optical Density (O.D) read from the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific) at absorbance of 595 nm. THY was used as a blank.

2.4.1.4 Discontinuous SDS-PAGE and Western Immunoblotting

Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting tests were carried out to confirm the presence of proteins of interest in the standard encapsulated type 2 (D39) and absence of specific proteins in the case of the mutant strain. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a method used to separate and/or identify proteins in a mixture according to their molecular size. SDS portion is a negatively charged detergent and is used to reduce proteins to their primary structure and coat them with uniform negative charges. When an electric field is applied across the gel, the proteins migrate toward the positive pole and depending on their size, each protein will move differently through the gel matrix: smaller proteins move through the matrix faster than large ones. Western immunoblotting is a method used to identify or detect a protein in a mixture of any number of proteins using an antibody that is specific to the protein while giving information about the size of the protein.

Each pneumococcus concentrated culture supernatant (containing 20µg of protein) was mixed with 12.5 µl of loading buffer [ddH_20 16µl, 0.5M Tris, pH 6.8 5 µl, 50% glycerol 8µl, 10% SDS 8µl, 2-β mercaptoethanol 2ul and Bromophenolblue] and the volume made up to 50ul with ddH_20 in eppendorf tubes and heated for 5 minutes at 95°C to denature the proteins. The samples (20 µl) were then loaded onto a 12% Bio-Rad Read Precast gel (Bio-Rad, UK) together with a Bio-Rad pre-stained standard and ran at 180V constant voltage for 40 minutes.

The proteins in the gel were then transferred to a nitrocellulose membrane (Bio-Rad) by electro blotting. Briefly, the electrophoresed gel was dipped in transfer buffer (1X Tris/Glycine/SDS buffer, Bio-Rad laboratories, GmbH, Munchen) and then laid flat on pre-wetted 2 layers of filter papers supported by a fibre pad resting on the cathode. Nitrocellulose membrane was placed on top of the gel and overlaid with 2 filter papers and a fibre pad. Any air bubbles that may have formed inside the sandwich were smoothed away with a pipette. The assembly was placed in the transfer cell with the black side facing the cathode (black electrode). The transfer was done at 0.3 A for 60 minutes.

The blots were blocked with 10ml of 3% BSA in PBST plus Sodium Azide (500ul 100X Sodium Azide+ 50 ml 3% BSA in PBST[PBST-250µl Tween 20 in 500ml 1X PBS]) for 1 hour at room temperature, followed by 4°C on a shaker overnight. The following day, the blocking solution was removed and the blot incubated with the mouse polyclonal antiserum to Ply (diluted 1 :4000[2ul anti-serum to Ply + 8 ml 1% BSA-PBST] gift from James Paton, Adelaide University, Adelaide, Australia) for 1 hour at room temperature with shaking. After washing with PBST, the blot were incubated with an anti-mouse IgG alkaline phosphatase conjugate (diluted 1 :1000[5ul conjugate + 5 ml 1% BSA-PBS]) for 1 hour at room temperature with gentle shaking on a rocker. The blots were washed as previously described and developed using an alkaline phosphatase substrate buffer containing NBT (Nitro-Blue Tetrazolium Chloride) and BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) at on a rocker at room temperature. NBT and BCIP yield an intense, insoluble black-purple precipitate when reacted with Alkaline Phosphatase. The colour reaction was stopped with deionised water once the protein bands had become clear.

Recombinant pneumococcal proteins

Recombinant pneumococcal proteins CbpA, PspA, PsaA and the genetic toxoid derivative of pneumolysin (PdB) were kindly provided by Dr James Paton, Adelaide, Australia. They were used at a final concentration of: CbpA – 1.5 μg/ml, PspA – 2 μg/ml, PsaA – 2 μg/ml and PdB – 2 μg/ml.

2.4.2 Diphtheria Toxoid

Diphtheria toxoid (D52.2; Conc- 1911Lf/ml; Purity- 2222 Lf/mg PN; Statens Serum Institut, Copenhagen, Denmark) was used at a final concentration of 2Lf/ml.

2.4.3 Control Antigens and Mitogens

Positive antigen controls and mitogens used in this study were:

i) *Mycobacterium tuberculosis* purified-protein-derivative (PPD RT50; Statens Serum Institut, Copenhagen, Denmark) at final concentration of 10μg/ml (unless stated otherwise). It was used as a positive control in Malawian setting because of universal neonatal BCG vaccination.

ii) Inactivated influenza vaccine (Split Virion) BP (2009/2010 vaccine; Sanofi Pasteur MSD Limited UK). It contains 15 μg of haemagglutinin (HA) of each of the following strains: A/Brisbane/59/2007(H_1N_1) - like strain (A/Brisbane/59/2007(IVR-48), A/Brisbane/10/2007(H_3N_2) – like strain (A/Uruguay/716/2007[NYMC X-175C]), B/Brisbane/60/2008 – like strain (B/Brisbane/60/2008).

iii) Phytohemaglutinin (PHA) (Sigma-Aldrich, St-Louis, MO) (an extract of red kidney beans that induces global activation of T cells) was also used as a positive control at 5μg/ml (unless stated otherwise).

iv) Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St-Louis, MO) and Ionomycin (ionophore produced by bacterium *Streptomyces conglobatus*) (Sigma-Aldrich, UK). PMA (10ng/ml) and Ionomycin (500ug/ml) were used together to stimulate intracellular production of cytokines.

v) *Staphylococcus* enterotoxin B (SEB) (Sigma-Aldrich) (exotoxin produced by *S. aureus*). It is a superantigen and causes non-specific activation of T cells resulting in polyclonal T cell activation and cytokine release (Krakauer 1999; Llewelyn and Cohen 2002). It was used at a final concentration of 1µg/ml

vi) Purified Tetanus Toxoid (NIBSC,UK) was used at a final concentration of 5µg/ml

vii) Pokeweed mitogen (PWM) (1/1000; Sigma), *Staphylococcus aureus* Cowan (SAC) (1/100 000; Calbiochem, Merck Biosciences, UK) and CpG ODN-2006 (final concentration 1µg/ml; InvivoGen, San Diego, CA) combined were used as polyclonal B cell activators (Crotty, Aubert et al. 2004).

2.4.4 Antibodies

Antibodies used in multicolour flow cytometry are summarised in Table 2.1(see below):

Table 2.1 | Staining panel for multicolour flow cytometry

Antibody	Fluorochrome(s)	Clone	Company
CD3	APC-H7	SK7	BD Pharmingen™
CD3	FITC	H1T39	BD Pharmingen™
CD3	PerCP	SK7	BD Biosciences
CD4	APC	RPA-T4	BD Pharmingen™
CD4	APC-H7	SK1	BD Biosciences
CD4	ECD	SFC112T4D11	Beckman Coulter
CD4	Pacific Blue™	RPA-T4	BD Pharmingen™
CD4	PerCP	SK3	BD Biosciences
CD8	APC-Cy7™	SK1	BD Pharmingen™
CD8	PerCP	SK1	BD Biosciences
CD25	FITC	2A3	BD Biosciences
CD28	PE	CD28.2	BD Pharmingen™
CD45RA	FITC	L48	BD Biosciences
CD45RA	PE	HI100	BD Pharmingen™
CD57	APC	HCD57	BioLegend
CD69	APC	L78	BD Biosciences
CD69	PC5	FN50	BD Pharmingen™
CD154	PE	TRAP-1	BD Pharmingen™
CD197(CCR7)	PE-Cy™7	3D12	BD Pharmingen™
CD197(CCR7)	APC-AlexaFluor® 750	3D12	eBioscience
Foxp3	PE	236A/E7	eBioscience
IFN-γ	FITC	B27	BD Pharmingen™
IFN-γ	APC	25723.11	BD FastImmune
IgG1, K	PE	MOPC-21	BD Pharmingen™
IL-2	APC	5344.11	BD Biosciences
IL-2	PE	MQ1-17H12	BD Pharmingen™
TNF-α	Alexa Fluor® 488	MAb11	BD Pharmingen™

2.5 Lymphocytes Phenotyping

2.5.1 T Cell Phenotyping

Careful measure or characterization of different cell types of the immune system such as central memory and immunoregulatory cells in well-characterized patients provides useful information on the utility of these cell subsets in immunity, regulation of the immune responses and immune exhaustion. The proportions of naive and memory T cells in whole blood were determined by flow cytometry on the basis of surface expression of CD45RA and CCR7(Sallusto, Lenig et al. 1999; Harari, Vallelian et al. 2004). Regulatory T cells (Tregs) were identified using a phenotypic definition involving coexpression of surface CD3, CD4 CD25 and intracellular Forkhead box p3 (Foxp3). Senescent cells were characterized by surface expression of either CD57 and CD28 or Programmed Death-1 (PD-1) (Brenchley, Karandikar et al. 2003; Warrington, Vallejo et al. 2003; Day, Kaufmann et al. 2006).

Whole blood: 200 ul of peripheral blood was aliquoted into appropriately labelled 12 x 75 mm FACS tubes (Naive/memory T cells, Tregs and Senescent T cells). The aliquots were stained extracellularly at room temperature in the dark for 10 minutes with a combination of antibodies specific to each of the three T cell phenotype groups. A combination of CD3 PerCP, CD45RA PE, CD8 APC-Cy7^{TM}, CD4 APC and CCR7 PE-Cy^{TM}7 antibodies all from BD Biosciences was used to stain for naive/memory T cells. CD3 PerCP, CD4 pacific blue, CD25 FITC antibodies (BD Biosciences) were used for Tregs, For analysis of senescent T cells, peripheral blood was stained with anti-CD3 FITC, anti-CD28 PE, anti-CD4 pacific blue, anti-CD57 APC and anti-CD8 PerCP or a combination of anti-CD4 PerCP, anti-CD38 FITC, anti-PD-1 PE and anti-CD57 APC all antibodies from BD Biosciences.

After surface staining, red blood cells were lysed with 3 ml of 1X BD FACS^{TM} Lysing solution (BD Biosciences) for 20 minutes at room temperature in the dark. Next, the cells were washed with 1X PBS at 700 x g for 7 minutes and the supernatant removed. The cell stained for naive/memory T cells and senescent T cells were resuspended in 2% paraformaldehyde (Sigma-Aldrich, UK) and kept at 4^0C until acquisition. The cells were surface-stained for Tregs were fixed and permeabilised using 400µl of freshly prepared 1X Foxp3 Fixation/Permeabilization working solution (ebiosciences, San Diego, CA) at 4^oC for 20 minutes. The cells were washed with 1X Perm wash (ebiosciences, San Diego, CA) by centrifugation at 700 x g for 5 minutes and the supernatant removed.

Thereafter, the cells were stained intracellularly with an anti-Foxp3 PE (ebiosciences) for 30 minutes at 4°C and then washed with 1X Perm wash and supernatant decanted. The cells were acquired on a Cyan^{TM} ADP flow cytometer (Beckman Coulter), configured to detect 11 parameters. Approximately 60 000 lymphocytes were acquired. Single stained cells and cells stained using all 5 fluorochromes were used to calculate the compensations. Automatic compensation was checked and adjusted manually where necessary. Data were analyzed with either FlowJo software (TreeStar Inc, Stanford University, FlowJo Africa scheme) or Summit software version 4.3.2 (Beckman Coulter). Lymphocytes were gated based on forward scattering (FSC) and side scattering (SSC) and CD3+, CD8-, and CD4+ cells were considered to be CD4 T cells. These CD4 T cells were analysed for Foxp3+ CD25^{high} cells (Tregs), CD45RA and CCR7 expression [naive/memory cells: T_{CM} (CCR7+ CD45RA-), T_{EM} (CD4+ CCR7-, CD45RA-) and naïve CD4 T cells (CCR7+ CD45RA+)] and CD57+CD28- cells or PD-1+CD38+ cells (senescent cells).

PBMCs after antigen stimulation: After stimulation with appropriate antigens, the cells were harvested into 12 x 75 mm FACS tubes and washed with 1X sterile PBS at 700 x g for 7 minutes and the supernatant decanted. The cell pellet was resuspended in 100 ul of 1X PBS (staining buffer) and stained with LIVE/DEAD® Fixable Violet stain (ViViD) (Invitrogen, UK) for dead cell discrimination at 4°C for 20 minutes. After washing as previously described, the cells were surface stained with CD4 ECD (Beckman Coulter), CD45RA FITC (BD Biosciences) and CCR7 Alexa Fluor® 750 (ebiosciences) for 15 minutes at 4°C. Next, the cells were washed with 2 mls of 1X PBS at 700 x g for 7 minutes. The cells were acquired on a Cyan^{TM} ADP flow cytometer (Beckman Coulter) and analysis performed with Summit software (version 4.3.2, Beckman Coulter). At least 100 000 lymphocytes were collected. Cell doublets were excluded using forward light scatter–area vs forward light scatter-height parameters. Lymphocytes stained positively for CD4 were analyzed for CD45RA and CCR7 expression.

2.5.2 B Cell Phenotyping

200ul of peripheral blood was aliquoted into a 5 ml FACS tubes (BD, UK) and stained with Fluorescein isothiocyanate (FITC)-, Phycoerythrin (PE)-, and Allophycocyanin (APC)-conjugated anti-human monoclonal antibodies binding to CD19, CD27, surface IgD (BD Biosciences) respectively for 10 minutes at room temperature in the dark. The red blood cells were then lysed with 3 ml of 1X BD FACS^{TM} Lysing buffer for 20 minutes at room temperature in the dark. The cells were washed with 2 mls of 1X PBS at 700 x g for 7 minutes and the supernatant decanted. The cell pellet was resuspended in 200ul of 2% paraformaldehyde (Sigma-Aldrich, UK). Data were acquired using acquired the Cell Quest Pro software (Becton Dickinson, version 5.2.1) on a four colour FACSCalibur flow cytometer (Becton Dickinson) and analyzed with FlowJo software (TreeStar Inc, Stanford University, FlowJo Africa scheme). At least 60 000 lymphocytes were acquired. Lymphocytes were gated based on characteristic forward and side scatter profiles. Then the cells stained positively for CD19 within the lymphocyte gate (B cells) were selected and analysed for CD27 and IgD expression [Marginal zone memory B cells (CD27+ IgD+), Germinal centre memory B cells (CD27+ IgD-) and naïve B cells (CD27- IgD+)].

2.6 CFSE Cellular Proliferation

Cellular proliferation is an essential feature of the adaptive immune response and a 5, 6-carboxylfluorescein diacetate succinimidyl ester (CFSE) flow cytometry based method provides a tool of assessing the proliferative activity of cells during an immune response. The assay relies on the ability of a highly fluorescent CFSE, to form highly stable bonds with intracellular and cell surface proteins by reaction with lysine side chains and other available amine groups. When cells divide, the fluorescent molecules are distributed equally to progeny cells resulting in a halving of the fluorescence of the daughter cells. Consequently, halving of the fluorescence intensity indicates each successive generation in a population of proliferating cells which can be easily tracked by flow cytometry (Mannering, Morris et al. 2003; Quah, Warren et al. 2007).

To identify dividing T lymphocytes following antigenic challenge, as previously optimised in this laboratory, 12 x 10^6 freshly isolated PBMCs were resuspended in 1 ml PBS and labelled with CFSE dye (final concentration 1.25 µM, Invitrogen) for 8 minutes at 37°C in the dark (kept free from excessive light to avoid photo bleaching). Next, 200ul of fetal bovine serum (Sigma-Aldrich, UK) was added to stop CFSE staining and the cells were washed at 700 x g for 5 minutes. The supernatant was discarded and the pellet was resuspended in 3 ml complete RPMI (RPMI 1640 supplemented with penicillin/streptomycin (concentration, 100 U/mL), L-glutamine (4 mmol/L), HEPES, (10 mmol/L; Sigma-Aldrich) and 2% (vol/vol) of heat-inactivated human AB serum (National blood Services, Blantyre, Malawi).

The cells were recounted and complete RPMI added to make a final cell concentration of 0.8 x 10^6 cells/ml. Thereafter, the cells (0.8 x 10^6/ml) were cultured in a 48-well flat bottom plate (Costar, Appleton Woods, UK) in the presence of appropriate antigens and controls for 8 days at 37°C in a 5% CO_2 incubator. On day 8, the cells were harvested into labelled FACS tubes and washed twice with 1 ml 1X PBS at 700 x g for 7 minutes. The cells were resuspended in 100 ul PBS (staining buffer), stained with anti-CD4-APC and anti-CD8-Percp (BD Biosciences), and incubated in the dark at 4°C for 15 minutes. After washing with 1 ml 1X PBS, the cells were fixed with 200 ul of a 2% paraformaldehyde (Sigma) and acquired using the Cell Quest Pro software (version 5.2.1) on a four-colour flow cytometer (BD FACSCalibur, BD Biosciences). 60 000 events in the lymphocyte gate were acquired.

Single-stained PBMC samples were used to periodically adjust fluorescence compensation settings on the FACSCalibur. Data were analysed with FlowJo software (TreeStar Inc, Stanford University, FlowJo Africa scheme) by initially gating on lymphocytes (including blasts). This was followed by gating on CD4+ or CD8+ $CFSE^{low}$ and $CFSE^{high}$ lymphocytes in a CD4+/CD8 versus CFSE plot.

2.7 Interferon-γ Enzyme-linked Immunospot (ELIspot) Assay

Interferon gamma Enzyme-Linked Immunospot (ELISpot) assay provides a sensitive means for quantifying antigen-specific T cells at the single cell level by detection of antigen-induced cytokine secretion (Mashishi and Gray 2002; Karlsson, Martin et al. 2003). The cytokine (IFN-γ) secreted by stimulated cells is captured locally by a monoclonal antibody that is immobilised on a nitrocellulose membrane.

The ELISpot assay was performed in collaboration with Mr David Mzinza as previously described by this laboratory (Mureithi, Finn et al. 2009). Briefly, Multiscreen HTS^{TM} 96-Well Filtration plates (Millipore, Ireland) were coated with 15 ug/ml anti-human IFN-γ mouse monoclonal antibody (1-D1K; Mabtech, Stockholm, Sweden) in sterile phosphate-buffered saline (PBS) for 4 hours at room temperature.

The plates were washed six times with 1X sterile PBS to remove unbound coating antibody and blocked for 1 hour with 200ul RPMI supplemented with 10% fetal calf serum (Sigma). PBMCs suspended in complete media were seeded at 0.5 x 10^6 cells/well in duplicate wells and were left unstimulated (negative control) or were stimulated with appropriate test antigens and controls for 18 hours at 37°C, 5% CO_2. 10μg/ml PPD (Statens Serum Institute) and/or 0.9μg/ml influenza vaccine, split-virion inactivated (2009/2010 vaccine; Sanofi Pasteur MSD Limited UK) were used as positive antigen controls and 5μg/ml PHA-L (Sigma-Aldrich, St-Louis, MO) as a mitogenic control.

After 18 hours of incubation, the plates were washed six times with 1X sterile PBS containing 0.05% TWEEN®20 (Sigma-Aldrich) followed by three times with sterile 1X PBS. 1 ug/mL of biotinylated anti–human IFNg antibody 7-B6-1 (MABTECH) diluted in 0.5% fetal calf serum plus 1X PBS was added to each well and the plates were incubated for 2 hour at room temperature. Next the plates were washed as before and incubated with 1μg/ml of streptavidin-alkaline phosphatase conjugate (Mabtech) for 1 hour at room temperature. The plates were washed six times with 1X PBS containing 0.05% TWEEN®20 followed by three times with sterile PBS and were developed using a chromogenic alkaline phosphatase substrate kit (Bio-Rad) for 15 minutes at room temperature. The plates were washed three times with running water to stop the reaction and then dried at room temperature in the dark. Spot forming cell numbers were counted using an ELISPOT plate reader (AutoImmuneDiagnostica, Vers. 4.0). The results were expressed as spot forming units (SFU) per million PBMC after subtraction of individual background values.

2.8 B Cell ELISpot

The B-cell ELISpot is a highly sensitive assay that allows at the cellular level, detection and enumeration of the total number of antibody secreting cells in a sample as well as those secreting antibodies to a specific antigen. In an antigen-specific setting, the assay can be performed in either of two ways, one where the antigen is immobilized on the ELISpot plate and one where the same antigen is instead used for detection. In this work, the former was used.

The ELISpot was performed as previously described (Crotty, Aubert et al. 2004) with modifications devised by Dr Olowadamilola H. Unuigbe-Iwajomo (PhD thesis, 2011). 2 $x10^6$ freshly isolated PBMCs were plated in a 24-well plate in 2ml RPMI 1640 medium supplemented with 10% fetal calf serum (Sigma-Aldrich, UK), 2 mM L-glutamine, 100 U/ml penicillin, 20 mM HEPES buffer and 100 ug/ml streptomycin (Sigma-Aldrich, UK) and polyclonally activated with a mix of the following: 1µg/ml phosphothiolated CpG oligodeoxynucleotide (CpG ODN 2006) (InvivoGen, San Diego, CA), 1/100,000 standardized pansorbin cell (SAC) (Calbiochem, Merck Biosciences, Nottingham, UK), and 1/1000 pokeweed mitogen extract (PWM) (Sigma). Cells were cultured for 6 days at 37°C, 5% CO2. A negative control well was cultured with media alone. In preparation for the ELIspot (on day 5 of cultured PBMCs), multiscreen HTS™ 96-Well Filtration plates (Millipore) were coated with appropriate antigens overnight at 4°C. A pool of 10 ug/ml PPD (Statens Serum Institute, Copenhagen, Denmark), 5µg/ml purified tetanus toxoid (TT) (NIBSC,UK) and 1µg/ml influenza vaccine (2009/2010 vaccine; Sanofi Pasteur MSD Limited UK) was used as a positive control and PBS as a negative control.

To detect all IgG and IgA secreting cells (used as assay controls), some wells were coated with 2µl/ml goat anti-human IgG antibody or 1µl/ml goat anti-human IgA antibody (Zymed, Invitrogen, Carlsbacl, CA). Samples were run in triplicate for each test antigen. On day 6 of the cell culture, the coated plates were washed six times with 200ul wash buffer (1X PBS containing 0.05% TWEEN 20[PBST]) followed by three 3 times with 1X PBS. The wells were blocked with RPMI-1640 plus 2% bovine serum albumin (BSA; Sigma) for 2 hours at 37°C prior to use. Stimulated cells were harvested and washed with RPMI media and 4 x 10^5 cells were added to the wells of the coated plate and incubated for 18 hours at 37°C, 5% CO_2.

After incubation overnight, the plates were washed as previously described. 1.6μg/ml biotinylated goat anti–human IgG detection antibody (A3188; Sigma-Aldrich, St Louis, Missouri) in 100ul of PBS/2% BSA was added to each well and the plate was incubated for 18 hours at 37°C, 5% CO_2. For total IgA, the wells were incubated in 0.5 μl/ml biotinylated goat anti–human IgA detection antibody (H140008; Caltag laboratories, Burklingame, CA)After incubation, the plates were washed six and 3 times with 200ul PBS-0.05% TWEEN 20 wash buffer and PBS respectively and developed with an alkaline phosphatase substrate buffer containing NBT (Nitro-Blue Tetrazolium Chloride) and BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) (Bio-Rad, UK) in the dark at room temperature for 10-20 minutes. The development mix was washed off the plates with gently running tap water, and dried at room temperature. Developed spots were counted with an AID ELISpot reader (AID software version, Vers. 4.0).

2.9 Induction and Flow cytometric analysis of CD154 expression

Flow cytometric measurement of CD154 provides the means of identifying CD4 T cells that may provide 'help' for the development of cell-mediated immune responses and T cell dependent antibody responses and identifying a wide array of antigen-responsive CD4 T cells (both cytokine –dependent and independent antigen-specific T cells)(Frentsch, Arbach et al. 2005; Bolton and Roederer 2009).

Two different approaches were assessed to study CD154 expression: conventional method (staining for cell-surface CD154 after stimulation) and coculture method (addition of CD154 to stimulation culture) as described elsewhere (Chattopadhyay, Yu et al. 2005; Wang, Cao et al. 2010) . Briefly, a minimum of 1.5 $x10^6$ or a maximum of 2 $x10^6$ freshly isolated PBMCs (depending on availability) were plated in a 96-well plate (Costar, Appleton Woods, UK) in 200 ul RPMI 1640 medium (Sigma-Aldrich, UK) supplemented with 2% heat inactivated AB human serum (National Blood Services, Blantyre, Malawi), 2 mM L-glutamine, 100 U/ml penicillin, 20 mM HEPES buffer and 100 ug/ml streptomycin (Sigma). For coculture method, an anti-CD154-phycoerythrin (PE) antibody was added to every well immediately before stimulation.

The cells (both conventional and coculture methods) were stimulated with appropriate test antigens and 4ug/ml PPD (Statens Serum Institute, Copenhagen, Denmark) as a positive antigen control and 0.5 µg/ml PHA-L (Sigma, St Louis, MO) as a mitogenic control for 18 hours at 37°C, 5% CO_2 incubator. Negative control wells were left unstimulated in all experiments. No costimulatory molecules were added. After stimulation, cells were harvested into appropriately labelled FACS tubes and washed with 1 ml of staining buffer (1X sterile PBS) at 700 g for 7 minutes. For the conventional method, cells were surface-stained with anti-CD3 fluorescein isothiocyanate (FITC), anti-CD4 peridinin chlorophyll protein (PerCP), anti-CD69 allophycocyanin (APC) and anti-CD154 phycoerythrin (PE) all from BD Biosciences for 15 minutes at 4^0C in the dark.

For the coculture method, the cells were surface-stained with anti-CD3 fluorescein isothiocyanate (FITC), anti- CD4 peridinin chlorophyll protein (PerCP), anti-CD69 allophycocyanin (APC). After staining, the cells were washed with 1ml 1X sterile PBS at 700 g for 7 minutes and resuspended in 2% paraformaldehyde. The cells were acquired using the Cell Quest Pro software (version 5.2.1) on a four-colour flow cytometer (BD FACSCalibur, BD Biosciences). Single-stained samples were used to periodically check the settings on the FACSCalibur. Data were analyzed with FlowJo software (TreeStar Inc, Stanford University, FlowJo Africa scheme) by initially gating on lymphocytes followed by CD3 then CD4+ cells. CD154 expression was examined on CD69+ CD4+ T cells.

2.10 CD4 ligation and CD154 expression

2 x10^6 freshly isolated PBMCs were plated in a 96-well plate in 200 ul 1X sterile PBS. An anti-CD4 domain 1 antibody (final concentration 10ug/ml [clone QS4120; Ancell, Bayport, MN]) was added to appropriate wells and the plate was incubated for 1h at 4°C as described elsewhere (Zhang, Fichtenbaum et al. 2004; Rutjens, Vermeulen et al. 2008). Thereafter, the cells were washed twice with 1ml sterile 1X PBS at 700 g for 7 minutes and resuspended in RPMI 1640 medium supplemented with 2% heat inactivated AB human serum (National Blood Services, Blantyre, Malawi), 2 mM L-glutamine, 100 U/ml penicillin, 20 mM HEPES buffer and 100 ug/ml streptomycin (Sigma). An anti-CD154-phycoerythrin antibody and anti-CD28/CD49d costimulatory antibodies (1µg/ml; BD Biosciences) were introduced into the cell culture immediately before stimulation.

Appropriate antigens were then added to the cells and the cultures were incubated for 18 hours at 37°C, 5% CO_2. SEB (final concentration 1μg/ml) was used as a positive control and No antigen (costimulantory antibodies only) was used as negative control. Next, the cells were harvest in FACS tubes and after washing with 1X sterile PBS, the cells were surface stained for 15 minutes at 4°C with anti-CD3 fluorescein isothiocyanate (FITC), anti- CD8 peridinin chlorophyll protein (PerCP), anti-CD69 allophycocyanin (APC) all from BD Biosciences. The cells were washed with 1ml 1X sterile PBS at 700 g for 7 minutes. Date were acquired on FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar Inc, Stanford University, FlowJo Africa scheme).

2.11 Intracellular Cytokine Staining for IFN-γ, IL-2 and TNF-α

Intracellular Cytokine Staining (ICS) assay (using flow cytometry) allows for detection of many different cytokines in T cells simultaneously following antigen challenge. The cells are stimulated with desired antigens in the presence of cytokine secretion inhibitor (Brefeldin A [BFA] or Monensin) and then fixed and permeabilised to be stained intracellularly with anti-cytokine fluorescent antibodies.

Two different methods were used to assess expression of various cytokines: a Brefeldin A based method to assess coexpression of IFN-γ, TNF-α and IL-2 and a Monensin based CD154 coculture method to assess coexpression of CD154 and IFN-γ and IL-2 (Chattopadhyay, Yu et al. 2006; Lamoreaux, Roederer et al. 2006). Briefly, a minimum of 1.5 $x10^6$ or a maximum of 2 $x10^6$ freshly isolated PBMCs (depending on availability) were plated in a 96-well plate in 200 μl RPMI 1640 medium supplemented with 2% heat inactivated AB human serum (National Blood Services, Blantyre, Malawi), 2 mM L-glutamine, 100 U/ml penicillin, 20 mM HEPES buffer and 100 μg/ml streptomycin (Sigma). For CD154 coculture method, an anti-CD154-phycoerythrin antibody was added to every well immediately before stimulation.

The cells were stimulated with appropriate test antigens and 10ug/ml PPD (Statens Serum Institute, Copenhagen, Denmark) and/or influenza vaccine, split-virion inactivated (2009/2010 vaccine; Sanofi Pasteur MSD Limited UK) as a positive antigen control and PMA (10ng/ml) plus ionomycin (1μg/ml)(Sigma, St Louis, MO) and incubated at 37°C in the presence of 5% CO2.

Brefeldin A (10 µg per ml) or Monensin (2 µM final concentration) all from BD Biosciences was added after 2 h of incubation and the plate was incubated for an additional 16 hr at 37^0C, 5% CO_2. Negative control wells were left unstimulated in all experiments. No costimulatory molecules were added. After stimulation, cells were harvested into appropriately labelled FACS tubes and washed with 1 ml of staining buffer (1X sterile PBS) at 700 g for 7 minutes and stained with ViViD (Invitrogen, UK) for dead cell discrimination (Figure 2.6) at 4°C for 20 minutes. After washing as previously described, the cells were stained at 4°C for 15 minutes with surface marker antibodies (BFA based method: CD4 APC-H7and CD8 PerCP all from BD Biosciences; Co-culture method: CD4 ECD [Beckman Coulter], CD3 APC-H7, CD69 PECy5™ all from BD Biosciences).

After staining, the cells were washed with 1ml 1X sterile PBS at 700 g for 7 minutes and permeabilised using 250 ml Cytofix/Cytoperm solution (BD Biosciences) for 20 minutes at 4°C. After washing twice with BD wash buffer, the cells were incubated for 30 min at 4°C with a cocktail of anti-human monoclonal antibodies the antibodies for intracellular cytokine staining (BFA based method: IFN-γ APC, TNF-α Alexa Fluor® 488 and IL-2 PE (BD Biosciences); Coculture method: IFN-γ FITC and IL-2 APC all from BD Biosciences).

Cells were washed again, resuspended in 2% paraformaldehyde. The cells were acquired on a Cyan™ ADP flow cytometer (Beckman Coulter), configured to detect 11 parameters. At least 100 000 lymphocytes were acquired. Cell doublets were excluded using forward light scatter–area vs forward light scatter-height parameters and dead cells discriminated by ViViD (Figure 2.6). Single stained cells and cells stained with all the fluorochromes were used to calculate compensations. Automatic compensation was checked and adjusted manually where necessary. Data were analyzed with FlowJo software (TreeStar Inc, Stanford University, FlowJo Africa scheme) or Summit 4.3.2 software (Beckman Coulter). Unstimulated cells were used to set cut-off gates for cytokines.

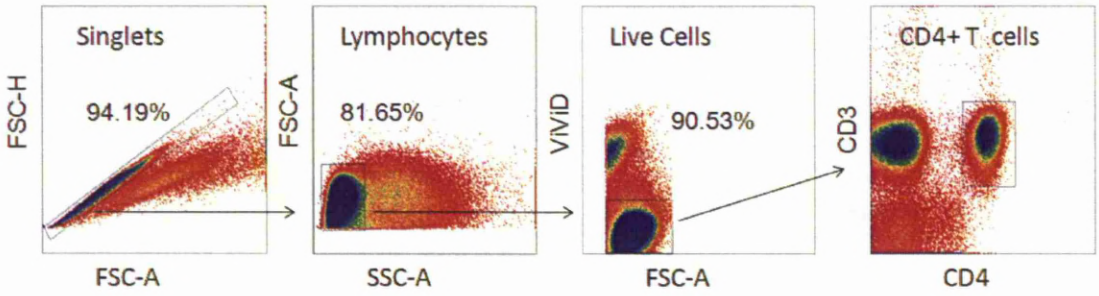


Figure 2.6 | Dead cell discrimination using Violet Viability dye (ViViD;LIVE/DEAD Fixable dead cell stain kit, Invitrogen). To exclude dead cells, PBMCs that have been stained with ViViD, were gated based on forward scatter height vs. Forward scatter area to identify singlets (single cells). Then lymphocytes were gated based on characteristic light-scatter properties. Live lymphocytes were gated based on forward scatter area without staining for the dead cell ViVID.

2.12 Multiplex analysis of cell culture supernatant cytokine concentration

Cytokines (IFN-γ, TNF-α, IL-10 and IL-2) in cell culture supernatant were quantified using a Bio-Plex cytokine assay (Bio-Rad Laboratories, Inc. Hercules, CA, USA; Human Grp 1 Plex panel [171-A11100]) according to the manufacturer's instructions in collaboration with Dr Sarah Glennie. Bio-Plex cytokine assay is a multiplex bead-based assay for measuring the levels of multiple cytokines in a single well of a 96 well microplate using as little as 50 ul aliquot of cell culture supernatant. The assay is designed in a capture sandwich immunoassay format. Each colour-coded 5.6 μm polystyrene bead set is coated with capture antibody specific for a particular cytokine. When a sample is combined with the antibody-coupled beads, the cytokine of interest is captured through a biochemical binding reaction. Captured cytokine is detected using a biotinylated detection antibody and streptavidin-phycoerythrin (S-PE).

Assay results are detected using a flow-cytometry based dual laser analyzer, the Luminex 200 (Bio-Rad, Hercules, CA). One laser is bead-specific and determines which cytokine is being detected. The other laser determines the magnitude of PE-derived signal, which is in direct proportion to the amount of cytokine bound [Bio-Plex™ Cytokine Instruction Manual, BIO-RAD].

Prior to the assay, the premixed lyophilized cytokine standard was reconstituted with 500 µl of standard diluents (stock concentration 32 000 pg/ml) and incubated on ice for 30 minutes. The standard stock was serially diluted with the standard diluent to generate 8 points for the cytokine standard curve.

A 96 –well sterile filter plate (supplied with the assay kit) was pre-wetted with 100 µl/well of assay buffer and removed the buffer by vacuum filtration (Bio-Rad Aurum™ Vacuum Manifold). 50 µl of premixed multiplex antibody-coupled beads was pipetted into each well plate (5,000 beads per well per cytokine) followed by vacuum filtration. The plate was washed twice with 100ul/well of Bio-Plex wash buffer using vacuum filtration. 50 µl of premixed standards or cell culture supernatant samples were added to each well. Blank control wells contained standard diluents. The plates was sealed with a plate sealing tape, covered with aluminium foil and shaken for 30 seconds at 1 100 rpm on a microplate shaker and incubated at reduced speed of 300 rpm for 30 minutes at room temperature.

After incubation, the buffer was removed by vacuum filtration and the plate washed 3 times as above. 25µl of 1X Bio-Plex detection antibody working solution (detector antibody conjugated to biotin) was added to each well and incubated for 30min with shaking (as described above). The plate was washed three times with 100µl/well with wash buffer using the vacuum filtration. This was followed by an incubation of 10 minutes with 50µl/well of 1X streptavidin Phycoerythrin (Streptavidin-PE) on a shaker as previously described at room temperature. After incubation, the plates were washed 3 times as previously described and the beads were resuspended in 125 µl of Bio-Plex assay buffer. The plate was read on the Luminex® 200™ analyzer (Bio-Rad Laboratories Inc, Hercules, CA). Raw data (mean fluorescence intensity, MFI) were analyzed using Bio-Plex Manager™ software (v 3.0). Unknown cytokine concentrations were calculated using a standard curve derived from recombinant cytokine standard supplied with the kit taking into account the supernatant dilution. All incubation steps were performed at room temperature and in the dark to protect the beads from light.

2.13 Statistical Analysis

Analysis and graphical presentation were done using Graphpad Prism Version 5 (Graphpad Software). Non-normally distributed data were analysed using non-parametric tests; Mann-Whitney *U* test for non-paired data and Wilcoxon test for paired data. Results are given as means with standard deviations (for parametric tests) while non-parametrically distributed data are quoted as medians with ranges. Differences after comparisons were considered statistically significant if they yielded *P* values less than 0.05.

CHAPTER 3 ASSAY OPTIMISATION AND ADAPTATION

3.1 Introduction

Epidemiological and experimental evidence suggests naturally-acquired antibodies to capsular and non-capsular components of *S. pneumoniae* (Rapola, Kilpi et al. 2001; Simell, Korkeila et al. 2001; Goldblatt, Hussain et al. 2005; Weinberger, Dagan et al. 2008), and CD4+ T cell responses to pneumococcal proteins, may mediate protection against pneumococcal disease (Zhang, Bagrade et al. 2007; Lu, Gross et al. 2008; Mureithi, Finn et al. 2009). HIV- infected persons however have a dramatically increased risk of infections with pathogens such as *Streptococccus pneumoniae, Mycobacterium tuberculosis* or *Pneumocystis jirovecii*. For the pneumococcus, HIV infection confers a 50-fold increased risk of invasive pneumococcal disease (Dworkin, Ward et al. 2001). Dramatic increased susceptibility to pneumococcal disease suggests defects in both T and B cell immunity to the pneumococcus are likely.

To better understand how T cell immunity to *S. pneumoniae* may be compromised in HIV-infected adults, it was first essential to identify parameters and develop or optimise assays to investigate underlying mechanisms. Assays that are usually used to assess immunity were discussed in Chapter 1 Introduction (section 1.14). A number of assays were used in this research project. These are: CFSE proliferation, IFN-γ ELISpot, IgG B cell ELISpot, Intracellular cytokine staining, Luminex cytokine analysis and CD154 expression assays. Except for the CD154 expression assay, the assays mentioned above were developed and optimised by others in this laboratory. CFSE cellular proliferation, IFN-γ ELISpot and B ELISpot assays were optimised or adapted for Malawi setting by Dr Sarah Glennie, Dr David Miles and Dr Oluwadamilola H. Unuigbe respectively. This chapter therefore describes the optimisation and adaptation of a novel assay for assessing surface expression of CD154 on CD4+ T cells (Chattopadhyay, Yu et al. 2005).

Flow cytometric measurement of surface CD154

Direct assessment of T helper cell responses specific by CD154 (a costimulatory cell surface protein that is expressed by activated CD4+ T cells) expression was first described independently by Frentsch M *et al.* (Frentsch, Arbach et al. 2005) and Chattopadhyay PK *et al* (Chattopadhyay, Yu et al. 2005). CD154 is transiently expressed on activated CD4+ T cells as it is rapidly internalised (and degraded) after surface expression (Roy, Waldschmidt et al. 1993; Yellin, Sippel et al. 1994). The studies that have therefore relied on conventional staining methods for detecting surface CD154 may overlook the dynamic nature of CD154 expression in the presence of CD40+ cells. Chattopadhyay PK *et al* however, described a co-culture method (where fluorescently labelled antibodies are added to stimulation culture) which allows immediate tagging of CD154 upon expression on the surface; subsequent internalisation of CD154 retains the conjugate rendering the cell fluorescent. The method was shown to identify a larger fraction of CD154 expressing CD4+ T cells compared to either the intracellular or the conventional surface staining method. Additionally, the assay is said to be compatible with intracellular cytokine staining allowing combined quantitative and qualitative *ex vivo* assessment of CD4+ T cell immunity for a defined antigen.

Recently, the co-culture assay was applied in a pulmonary tuberculosis study to understand the production and dynamics of memory T cell subsets during tuberculosis. (Wang, Cao et al. 2010). This assay however has not been applied to study the human response to pneumococcal antigens or for longer *in vitro* stimulation time without using costimulatory antibodies. Previous studies have reported impairment of CD154 expression in CD4+ T cells of HIV-infected individuals in response to polyclonal stimuli (Zhang, Fichtenbaum et al. 2004; Zhang, Lifson et al. 2006; Subauste, Subauste et al. 2007) and some pathogens (Subauste, Wessendarp et al. 2004). In order to assess the effect of HIV on the ability of pneumococcal-specific T cells to provide CD154-mediated help, the co-culture method for detecting surface-expressed CD154 molecues, was optimised for use in an African setting.

3.1.1 Rationale and Aims

This part of the research project aimed to develop, optimise and adapt appropriate assays to assess T helper cell responses. The main objective was to assess and compare different methods of assessing pathogen-specific induction of cell-surface CD154 in CD4+ T cells with the goal of identifying an assay that can be optimised and adapted for use in an African setting. Other objectives were to: i) batch test AB human sera to be used in cell culture assays in this research project; ii) characterise pneumococcal concentrated culture supernatant and iii) determine the time course of CD154 expression on CD4+ T cells following stimulation with pneumococcal protein antigens.

3.2 Materials and Methods

Samples collection and Processing (section 2.3)

- Venous Blood and PBMCs Isolation (sections 2.3.1 and 2.3.2)

Antigens used (section 2.4)

- Pneumococcal strains (section 2.4.1)
  - Preparation of pneumococcal culture supernatants (section 2.4.1.1.)
  - Determination of Colony Forming Units per ml (section 2.4.1.2)
  - Bradford Protein Assay (section 2.4.1.3)
  - Discontinuous SDS-PAGE and Western immunoblotting (section 2.4.1.4)

Control antigen and Mitogen used (section 2.4.3)

- *M. tuberculosis* purified-protein-derivative (PPD) (section 2.4.3i)
- PMA and Ionomycin (section 2.4.3ii)

Human AB sera batch testing using CFSE proliferation assay (section 2.6)

Induction and Flow cytometric analysis of CD154 expression (section 2.9)

Intracellular Cytokine Staining (IFN-γ and IL-17) (section 2.11)

3.3 Results

3.3.1 Human AB Sera batch testing

It is a well-established practice in this laboratory that where possible all cell culture assays should be carried out in media supplemented with AB human serum instead of foetal calf serum (FCS). In fact, previous work done by this group on T cell memory response to pneumococcal protein antigens in an area of high pneumococcal carriage and disease used AB human serum (Mureithi, Finn et al. 2009). Our experience with FCS is that it tends to give high background especially in assays involving long term culture. Therefore, all cell culture assays (except for T and B cell ELISpot assays) used in this project were carried out in media supplemented with heat inactivated AB human serum.

Three batches of AB human serum were donated to this research project by the National Blood Services of Malawi. The sera were screened by the National Blood Services for infectious agent such as HIV and hepatitis before they were given to the research project. Serum is an extremely complex mixture of plasma proteins, growth factors and hormones hence it is commonly used as a supplement in cell culture. The components of serum may vary according to origin, nutritive conditions and processing and because of these natural variations the three sera obtained from the National Blood Services were batch tested using CFSE (Invitrogen) proliferation assay. PBMCs isolated from blood collected from willing and consenting HIV negative adults from MLW clinical research programme, were labelled with 1.25 μM CFSE and stimulated with tuberculin 10 μg/ml PPD (Statens Serum Institut, Copenhagen, Denmark) or left unstimulated for 8 days in media supplemented with AB human serum and CD4+ T cells analysed for proliferation by flow cytometry. *M. tuberculosis* PPD was used as stimulant because it was more likely to give a response in Malawian setting because of universal neonatal BCG vaccination.

CD4+ T cells proliferated in all the three heat inactivated AB human sera (2%) in response to PPD (Figure 3.1). The cells proliferated slightly more in human serum S216 (12.18%, background removed) compared to sera S4383 and S5210 which recorded 11.17% and 7.44%, respectively. Cells however proliferated slightly more when the cell culture was supplemented with 10% AB human serum (14.34%) (Figure 3.2). Since the difference between 2% AB human serum and 10% human serum was not substantial subsequent cell culture assays were carried out using 2% pooled supportive AB human sera.

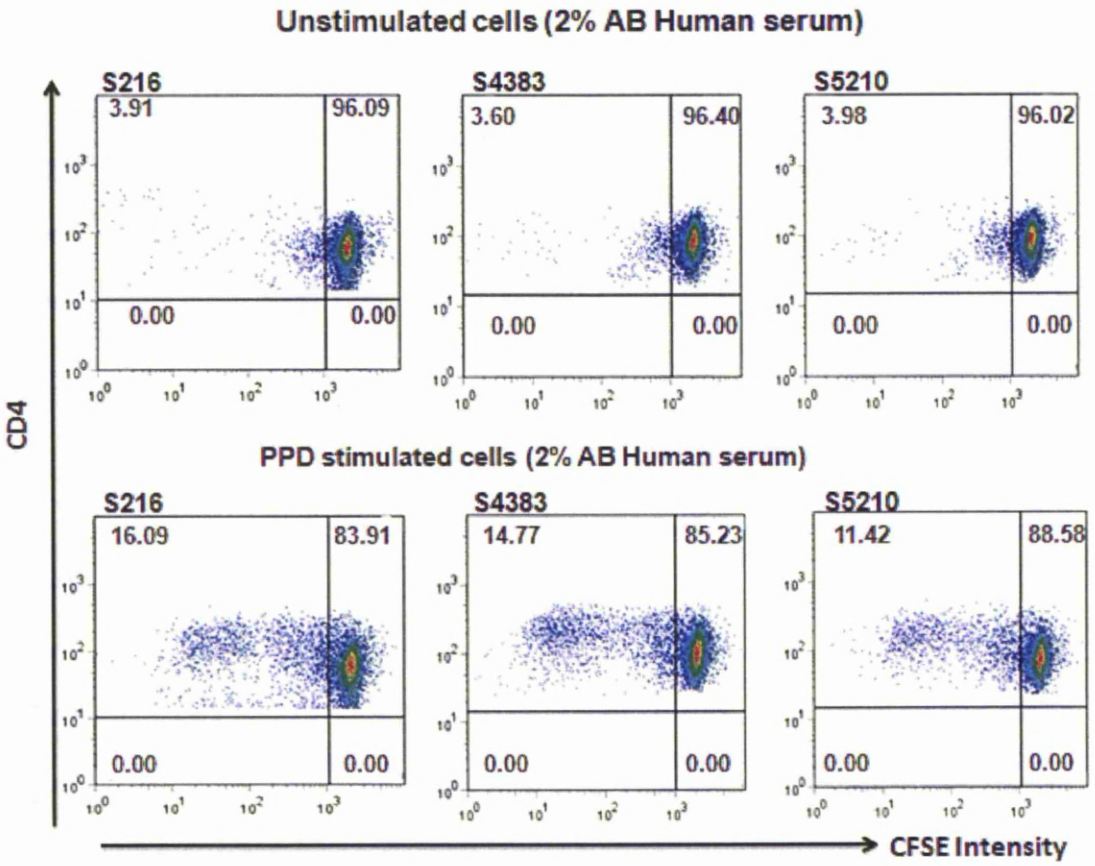


Figure 3.1 | CD4+ T cells proliferated in all AB human sera (2%). PBMCs were were labelled with 1.25 µM CFSE and stimulated with 10ug/ml purified protein derivative (PPD) or left unstimulated for 8 days. The cells were then stained with anti-CD4 monoclonal antibody and proliferation of CD4+T cells measured by flow cytometry. Cells in the upper left quadrant in each plot are indicative of proliferation. CD4+ T cells proliferated in all the three sera: S216-12.18%, S4383-11.17% and S5210- 7.44% (background subtracted). Results representative of three separate experiments.

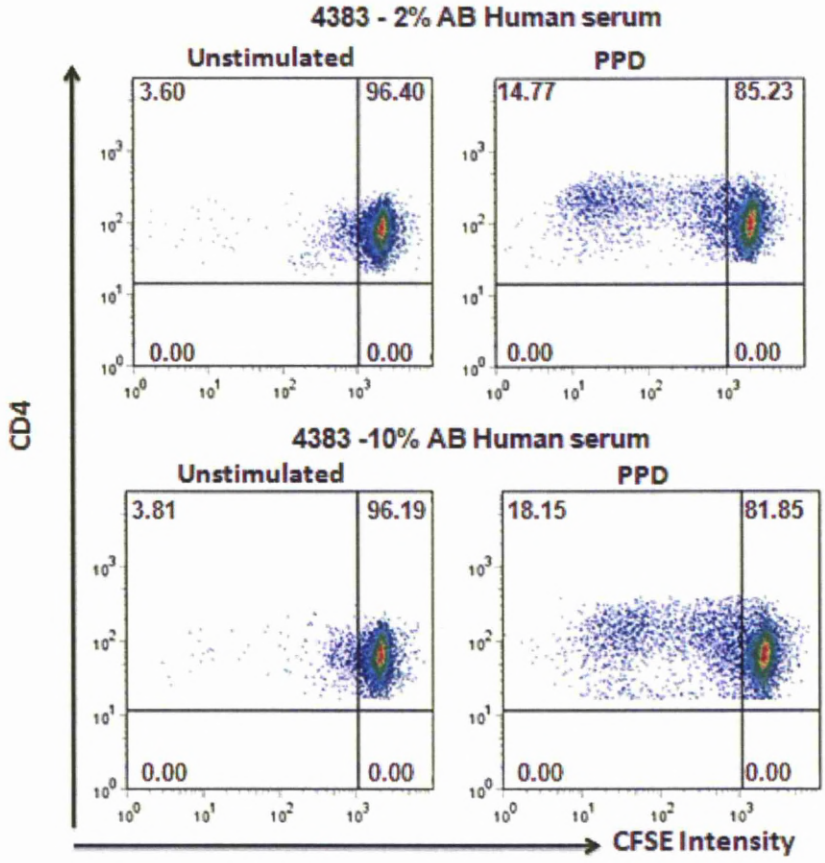


Figure 3.2 | CD4+ T cells proliferated in AB human sera; 2% versus 10% AB human serum. PBMCs were labelled with 1.25 uM CFSE and left unstimulated or stimulated with 10ug/ml purified protein derivative (PPD) for 8 days. The cells were stained with anti-CD4 monoclonal antibody and proliferation of CD4+T cells measured by flow cytometry. Cells proliferated slightly more in cell culture supplemented with 10% AB human serum (14.34%) compared with 2% AB human serum (11.17%), background subtracted. Results representative of three separate experiments.

3.3.2 Optimization of CD154 (CD40L) detection

3.3.2.1 Detection of surface CD154 expression by CD4+ T cells by conventional and co-culture methods.

Two different methods (the conventional method [staining for cell-surface CD154 after stimulation] and the co-culture method were used to detect membrane CD154 by flow cytometry (Chattopadhyay, Yu et al. 2005; Koguchi, Thauland et al. 2007). PBMCs from HIV negative subjects were stimulated with 10 ng/ml PMA (Sigma-Aldrich) plus 1μg/ml ionomycin (Sigma-Aldrich) or left unstimulated for 6 hours. For the co-culture method, a fluorescent antibody to CD154 was added before stimulation (Figure 3.3). After stimulation, the cells from the conventional method were stained with antibodies to CD3 (lymphocytes), CD4 and CD154 while the cells from the co-culture method were stained with antibodies to CD3 and CD4 (Figure 3.3). The cells were then analysed by flow cytometry and the level of CD154 staining was assessed on gated CD4+ T cells.

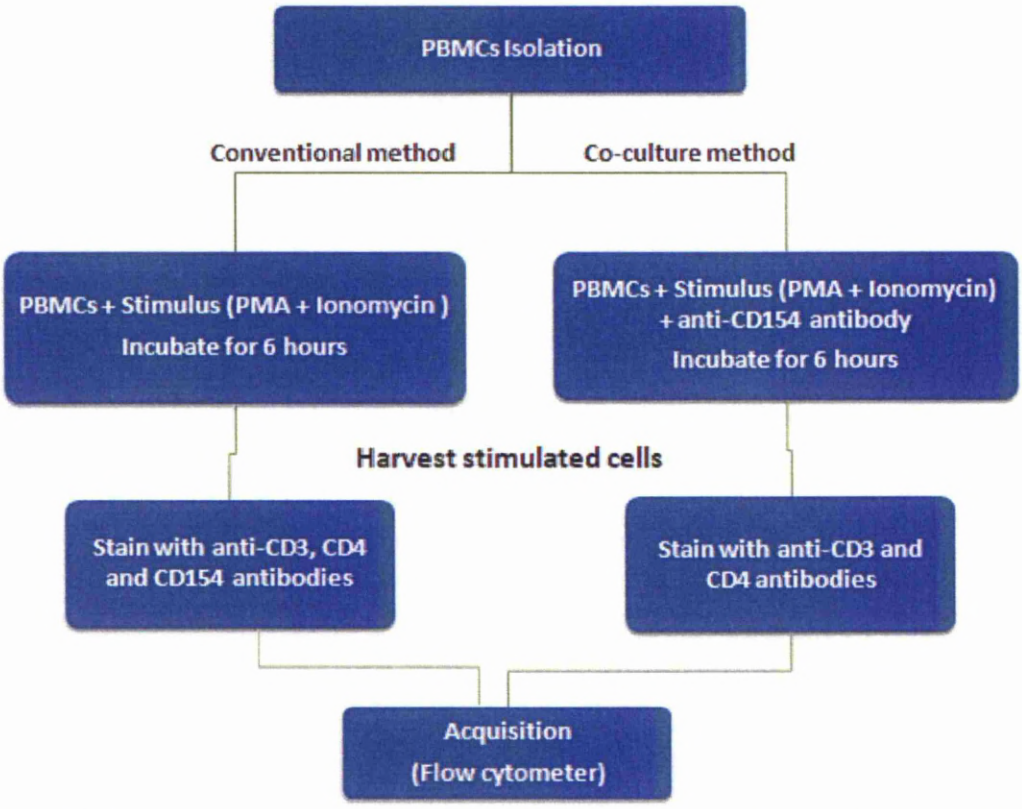


Figure 3.3 | Schematic summary of the methods used to detect membrane CD154 on CD4+ T cells.

As expected, the unstimulated cells from both methods did not express CD154 (Figure 3.4). The co-culture method identified a larger fraction of CD4+ T cells responding to PMA plus ionomycin stimulation (81.35% of CD4+ cells were CD154+, background subtracted) compared with the conventional surface staining method (64.94% of CD4+ T cells were CD154+).

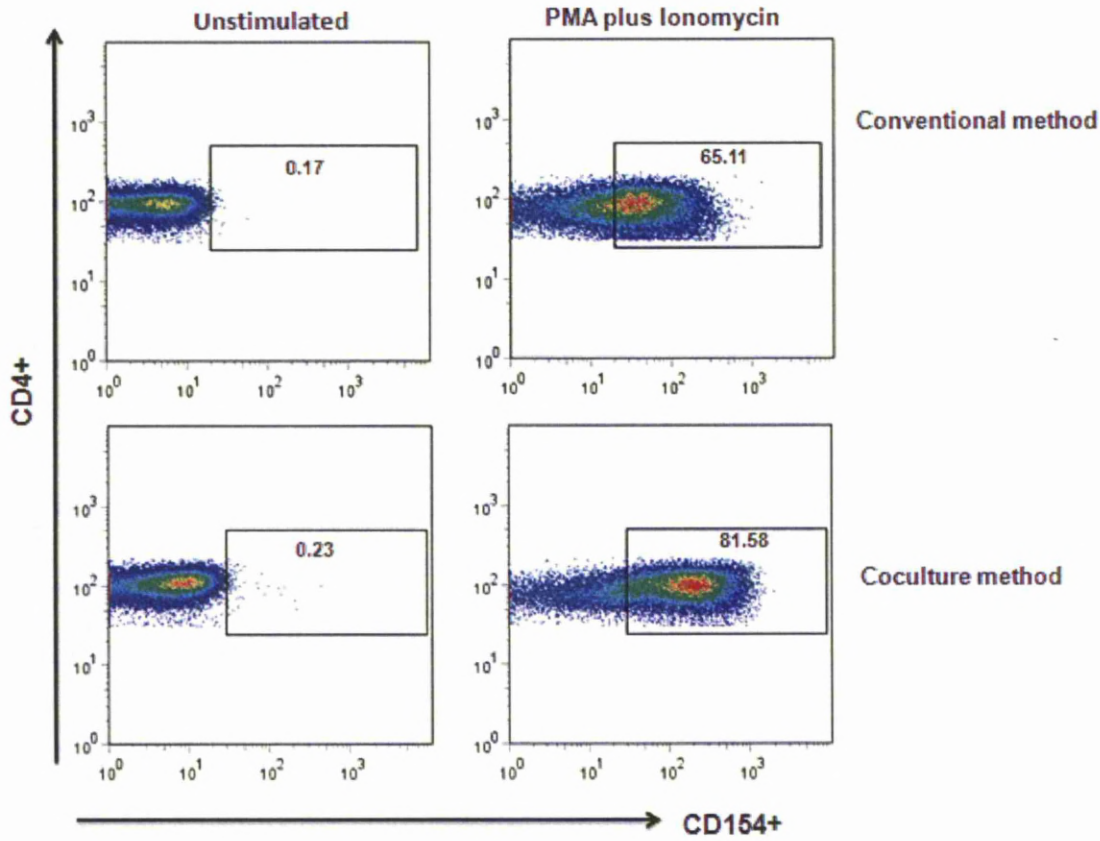


Figure 3.4 | Co-culture method identifies a larger fraction responding CD4+ T cells. PBMCs were stimulated with 10 ng/ml PMA plus 1ug/ml ionomycin or left unstimulated for 6 hours. For the co-culture method, an antibody to CD154 was included in the culture during stimulation. After stimulation, the cells for the conventional method were surface-stained with antibodies to CD3, CD4 and CD154 while the cells for the co-culture method were surface-stained with antibodies to CD3 and CD4. The level of CD154 staining was assessed on CD4+ T cells. The numbers inside the gates represent the percentage of CD4 T cells expressing CD154. The plots are representative of three separate experiments.

3.3.2.2 No uptake of the isotypic antibody in the co-culture method

CD154 expression is highly transient. CD154 is rapidly internalized after surface expression and so is the CD154-antibody complexes formed during stimulation in co-culture method. It was important to confirm, however that there is no uptake of the unbound antibody in the co-culture method. This was addressed by incubating cells with an isotopic antibody (PE-labelled anti-IgG mAb). The isotopic antibody was used at the same concentration as the antibody to CD154 (0.25ug/test). No PE staining was detected in the stimulated cells incubated with the isotypic antibody (Figure 3.5) indicating that there was no nonspecific uptake of the isotypic antibody. Thus, in the co-culture method it is only the CD154-antibody complex that is internalised. The co-culture method therefore was chosen as the method to use in subsequent experiments.

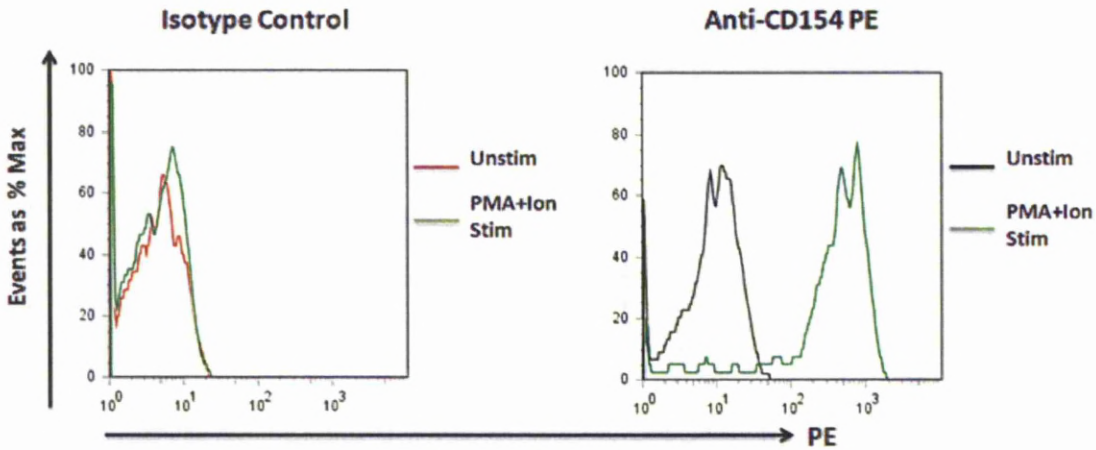


Figure 3.5|No nonspecific uptake of isotypic PE antibody in coculture method. PBMCs were stimulated or left unstimulated with 10ng/ml PMA plus 1ug/ml ionomycin for 6 hours. An equal concentration of anti-CD154 monoclonal antibody (mAb) and PE-labelled anti-IgG mAb (0.25ug/test-IgG1, κ isotype control, BD Pharmingen) was included in the culture during stimulation. The plots show the levels of PE staining gated on CD4+ T cells. The plots are representative of three separate experiments.

3.3.2.3 Monensin failed to enhance detection of CD4+CD154+ T cells

It has been reported that CD154-antibody complexes (in co-culture method) are unstable (unless monensin is added), leading to detection of fewer antigen responsive cells (Chattopadhyay, Yu et al. 2006). Therefore, we evaluated whether the presence of monensin in the culture during stimulation will lead to identification of more CD4+ CD154+ T cells compared to when monensin is not included in the culture (Fig. 3.6). Contrary to previous reports, adding monensin to the cell culture did not lead to detection of more CD154 expressing CD4+ T cells: 81.29% CD4+ CD154+ T cells (background subtracted) were detected in the absence of monensin and 80.85% CD4+CD154+ T cells were detected when the cells were incubated with monensin.

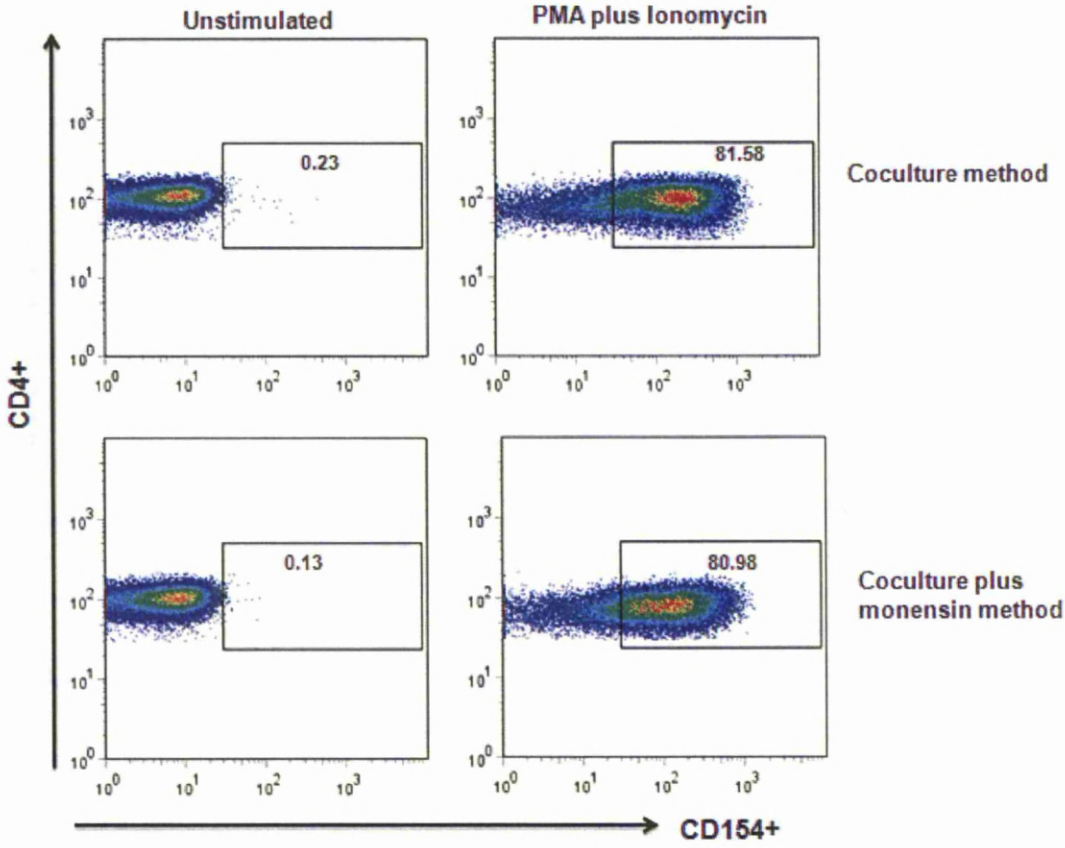


Figure 3.6|Monensin failed to stabilise CD154-antibody complexes. PBMCs were stimulated with PMA plus ionomycin or left unstimulated for 6 hours. An anti-CD154 monoclonal antibody (mAb) and monesin (2 μM) were included in the culture during stimulation. The levels of CD154 staining was gated on CD4+ T cells. The plot show the levels of CD154 staining gated on CD4+ T cells. The plots are representative of 3 separate experiments.

3.3.2.4 Monensin inhibited extracellular secretion of IFN-γ and IL-17

Monensin is usually used in intracellular cytokine staining assays to interrupt the maturation of cytokine processing and thus inhibit their extracellular secretion (Betts, Brenchley et al. 2003; Lamoreaux, Roederer et al. 2006). This leads to accumulation of cytokine in the cell, therefore aiding antibody detection. Since including monensin in the cell culture did not enhance the detection of CD154 expressing CD4+ T cells, we evaluated the ability of the monensin used in the previous experiment (section 3.3.2.3) to inhibit extracellular secretion of cytokines IFN-γ and IL-17 following stimulation with PMA plus ionomycin for 6 hrs. Brefeldin A (BFA [10 µg/ml] cytokine secretion inhibitor) was used as a control. The secretion inhibitors were added after 1 hour of stimulation. There was inhibition of extracellular secretion of cytokines (Figure 3.7) in the presence of monensin as well as in the presence of BFA.

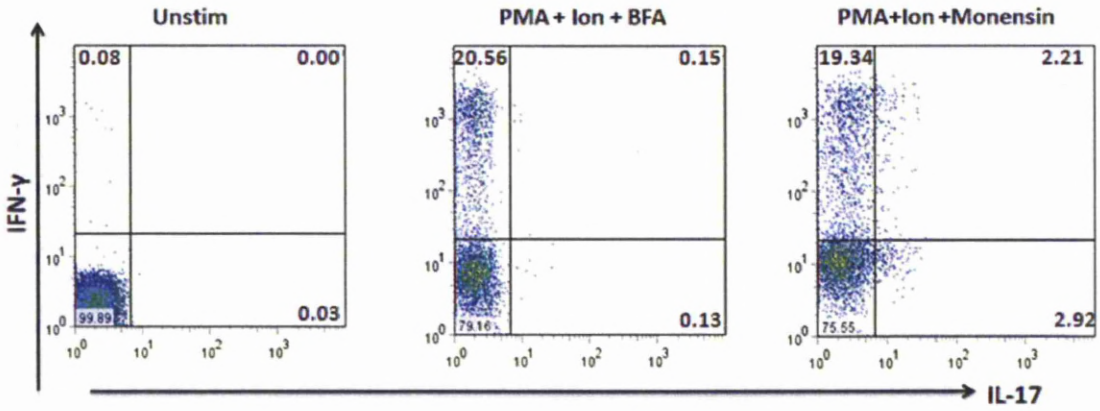


Figure 3.7|Monension inhibited extracellular secretion of IFN-γ and IL-17. PBMCs were stimulated with PMA plus ionomycin or left unstimulated for 6 hours in the presence or abscence of secretion inhibitors (brefeldin A (10 µg/ml or monensin [2 uM]). Secretion inhibitors were added after 1 hour of stimulation. Intracellular IFN-γ and IL-17 expression was analysed among CD4+ T cells. In presence of BFA, 20.24% CD4+ T cells expressing IFN-γ only were detected while in presence of monension 19.26% CD4+ T cells expressing IFN-γ only expressing CD4+ T cells were detected. The plots are representative of 3 separate experiments.

3.3.3 Characterisation of the pneumococcal concentrated culture supernatant

This project made use of pneumococcal culture supernatants prepared from a standard encapsulated type 2 (D39) wild type (WT), and an isogenic Ply-deficient mutant strain (Chapter 2: Materials and methods, section 2.4.1). Each strain was cultured in 5% CO_2 at 37°C to exponential phase (optical density of ~0.4 at 620nm; ~ 10^8 cfu/ml) (Figure 3.8). The culture supernatants were harvested by centrifugation (3000 x g for 30 minutes), filtered and the protein concentration of the pneumococcal concentrated culture supernatants (pneumoCCS) determined by the Bradford protein assay as described in Chapter 2 materials and methods (section 2.4.1).

Discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western immunoblotting were used to demonstrate that the pneumolysin mutant supernatant lacked pneumolysin. The western immunoblot was visualised using mouse anti-sera to pneumolysin (Ply). The Western immunoblot confirmed that the pneumococcal culture supernatants derived from a standard encapsulated type 2 (D39) *S. pneumoniae* strain contained Ply but Ply was missing in the mutant culture supernatant (Figure 3.9). The antiserum to Ply reacted with the wild type at ~53 kDa (Figure 3.9, *lane B*). A cross-reacting band is also visible at ~70 kDa on both blots.

Work done in this laboratory by Dr Marianne Mureithi (as part of her PhD, 2010) showed that heat-modification (56°C for 30 minutes) of pneumococcal concentrated culture supernatant enhances the magnitude of the pneumococcal T cell responses probably because at 56°C heat denatures toxic pneumococcal proteins (and in principle without affecting their immunogenicity) such as pneumolysin which was observed to be toxic to T cells in its native form but elicited a robust T cell response after heat-modification. Therefore, the pneumococcal concentrated culture supernatants were heat-treated at 56°C for 30 min before use.

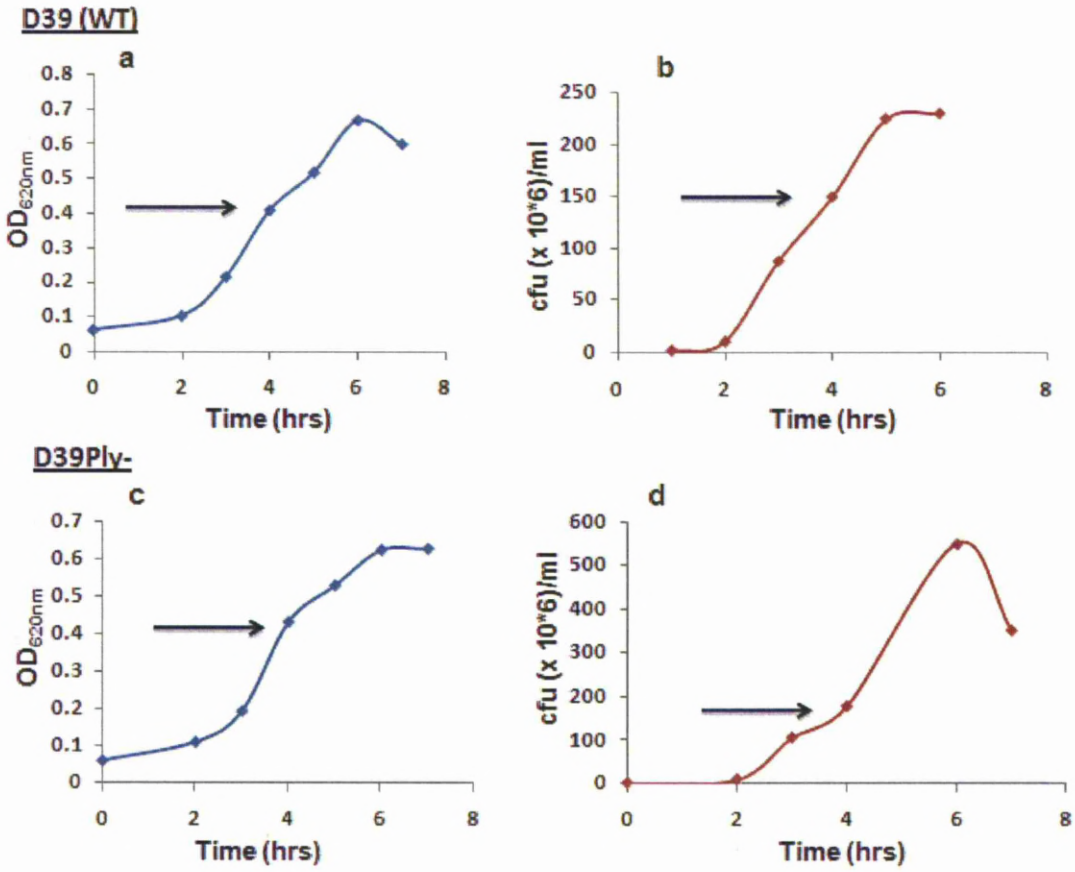


Figure 3.8|Growth profile of encapsulated type 2 (D39) wild type (WT) and an isogenic pneumolysin (Ply) -deficient pneumococcal mutant strain. Pneumococcal strains were cultured in Todd-Hewitt broth supplemented with 5% yeast extract in 5% CO2 at 37°C. **a,c|**Growth curves of encapsulated type 2 (D39) wild type and an isogenic pneumolysin (Ply) – deficient mutant strain respectively (at optical density of 620nm [OD_{620}]). **b,d|**Growth curves of encapsulated type 2 (D39) wild type and an isogenic pneumolysin (Ply) – deficient mutant strain respectively, based on colony-forming units (cfu/ml). → indicates that growth termination was at the exponential phase of ~ 0.4 at 620 nm; ~ 10^8 cfu/ml

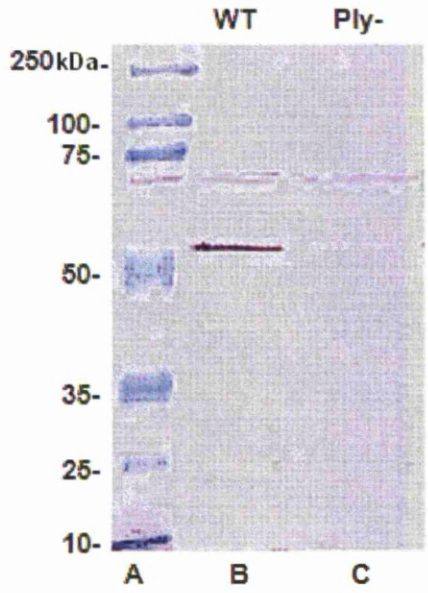


Figure 3.9|Western Immunoblot. Western immunoblot analysis of the concentrated culture supernatant of *S. pneumoniae* and visualised using mouse anti-serum to pneumolysin (Ply): **Lane A|** The molecular mass marker. **Lane B|** Pneumococcal culture supernatant derived from a standard encapsulated type 2 (D39[WT]) **Lane C|** Pneumococcal culture supernatant derived from an isogenic pneumolysin-deficient mutant (Ply-). 30 µl/20µg of protein was loaded into each well.

3.3.4 Time course of pneumococcal antigen induced CD154 expression

Having prepared and characterised pneumococcal concentrated culture supernatant, we investigated the kinetics of CD154 expression following stimulation with pneumococcal protein antigens using the co-culture method to determine the optimal time point for detection of CD154. Work done in this laboratory by Dr Marianne Mureithi (as part of her PhD work, 2010) showed that T cell responses (as measured by Thymidine incorporation proliferation assay), observed following stimulation with pneumococcal concentrated supernatant were associated to pneumococcal antigens present in the culture supernatant, and not to the culture Todd-Hewitt broth supplemented with 0.5% yeast extract (THY). No T cell responses were detected in response to THY-broth above background (RPMI 1640 medium supplemented with penicillin, L-glutamine and heat-inactivated human AB serum). Therefore, unstimulated cells in this project, were cultured in RPMI 1640 medium supplemented with penicillin, L-glutamine and heat-inactivated human AB serum. This approach has been used extensively in the work published by this group (Zhang, Bernatoniene et al. 2006; Mureithi, Finn et al. 2009).

To determine the optimal time point for detection of CD154, PBMCs were incubated with a fluorescently conjugated CD154 specific antibody and stimulated with 8μg/ml pneumoCCS (D39WT), or left unstimulated at 37°C, 5% CO_2. No costimulatory molecules (e.g. anti-human CD28 and anti-human CD49d antibodies) were used. The cells were removed from culture at various time points (after 6, 12, 18 and 24 hours of stimulation) to assess CD154 expression. After stimulation, the cells were stained with antibodies to CD3 and CD4 and analysed by flow cytometry.

In accordance with previous reports which have noted high levels of background CD154 staining in the absence of antigens (Chattopadhyay, Yu et al. 2005; Koguchi, Thauland et al. 2007), we observed some background CD154 staining in unstimulated cells which levelled off by 24 hours (Figure 3.10). There was a sequential increase in CD154 expression in stimulated cells with peak expression of CD154 detected with 18 hours of stimulation and maintained at 24 hours of stimulation (Figure 3.9; 6 hr- 0.040, 12 hr -1.733, 18 hr-3.207 and 24 hr-3.165). Based on this observation, 18 hour stimulation was selected as the optimal time point for detection of CD154 and there subsequent experiments used 18 hour stimulations.

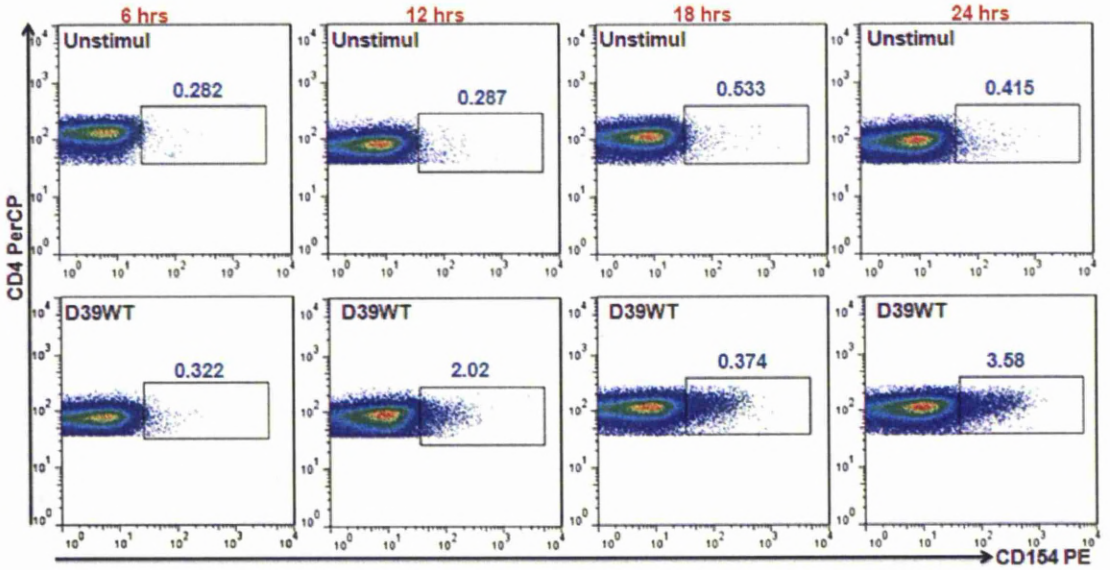


Figure 3.10|Time course for CD154 staining after stimulation with pneumoCCS (D39WT) as measured coculture method. PBMCs were stimulated with 8µg/ml pneumoCCS (D39WT) or left unstimulated at 37°C, 5% CO_2 and removed from culture at various time points: 6 hr stimulation, 12 hr stimulation, 18 hr stimulation and 24 hr stimulation. Anti-CD154 antibody was introduced into the cell culture immediately before stimulation and CD154 expression analysed among CD4+ T cells. A plateau of CD154 expression was reached 18 hours after stimulation. The numbers in **blue** represent the percentage of CD154 expressed on CD4+ T cells. The plot is representative of three (3) separate experiments.

3.4 DISCUSSION

HIV-infected persons are at increased risk of IPD compared with HIV negative individuals, suggesting defects in both T and B cell immunity to *S. pneumoniae* are likely. To investigate the influence of HIV infection on pneumococcal T cell immunity, there was need to optimise and adapt *in vitro* assays that would be used in this research project. Since it is common practice in this laboratory to use AB human serum in cell culture assays, AB human serum provided by the National Blood Services of Malawi was batched tested to determine whether it could be used in this research project and if so at what concentration. The testing was done using a CFSE proliferation assay optimised by others in this laboratory. We were able to show that PPD could elicit robust proliferative responses in PBMCs cultured in media supplemented with even 2% AB human serum. Therefore, subsequent experiments used 2% pooled supportive AB human sera.

Consistent with data from elsewhere (Chattopadhyay, Yu et al. 2005; Koguchi, Thauland et al. 2007), data herein show that the co-culture method identifies a larger fraction of CD4+ T cells expressing CD154 compared to the conventional surface staining method. Although, Chattopadhyay et al have shown that the inclusion of monensin in the cell culture (co-culture method) leads to detection of more CD4+ T cells that express CD154 compared to when monensin is not included (Chattopadhyay, Yu et al. 2006), we were unable to confirm this in our experiments..

Having identified the co-culture method as the appropriate method for measuring surface expression of CD154 on CD4+ T cells, the method was used to determine i) whether pneumococcal concentrated culture supernatants (pneumococcal protein antigens) can induce expression of CD154 on CD4+ T cells and ii) the optimal time point for detection of CD154 in the CD4+ T cells. Data herein show that pneumococcal protein antigens induced CD154 expression. Little is known about the role CD154 in cellular immunity to *S. pneumoniae.* Nonetheless, data from mouse and humanised mouse studies show that CD154 plays a role in the generation of antibodies to pneumococcal proteins and even to capsular polysaccharides (Jeurissen, Billiau et al. 2006; Moens, Wuyts et al. 2008).

Data presented in this chapter also showed peak expression of cell-surface CD154 with 18 hour of pneumococcal antigen stimulation. Therefore, subsequent experiments used 18 hour stimulations which was consistent with other studies which have reported 18 hour of antigen stimulation as the optimal time for detection of CD154 (Subauste, Wessendarp et al. 2004).

In conclusion, this chapter has shown that robust T cell responses to specific antigens can be generated when the cells are cultured in media supplemented with 2% AB human serum. This study has also established that CD154 can be efficiently detected by incubating cells with a fluorescent antibody to CD154 during stimulation. Having accomplished this, the CD154 co-culture method and other appropriate assays developed in this laboratory were subsequently applied in prospective and longitudinal studies of human immunity in Malawi to investigate the impact of HIV on naturally acquired cellular immunity to pneumococcal antigens (chapter 4), immune reconstitution of cellular and humoral immunity to pneumococcal antigens (Chapter 5) and vaccine induced immunity (chapter 6).

CHAPTER 4 Cross-sectional study of pneumococcal immunity in HIV infected Malawian adults

4.1 Introduction

HIV infection, with or without progression to acquired immunodeficiency syndrome (AIDS), dramatically increases the risk of pneumococcal invasive disease (IPD) and probably pneumococcal colonisation (Klugman, Madhi et al. 2007; Gill, Mwanakasale et al. 2008) suggesting that natural immunity to the pneumococcus (which is thought to rely on antigen-specific T and B cell memory) is susceptible to HIV-mediated immune dysregulation. In order to better understand the immunological basis for the high incidence of IPD in HIV-infected adults even in individuals with relatively good CD4 T cell counts, a study was designed to investigate pneumococcal-specific T cell mediated responses or T cell function in asymptomatic HIV infected Malawian adults.

4.1.1 T cell immunity

The pool of T cells found in the periphery consists of cells with diverse histories of engagement with antigens and distinct functional properties. After a first encounter with cognate antigens, naive T cells proliferate and differentiate into effector cells (T_{EM}) which can produce cytokines resulting in pathogen elimination (Bernasconi, Traggiai et al. 2002). Effector cells however have limited proliferative capacity and traffic to disease sites. Although the majority of the effector cells die, some survive to become long-lived memory T cells capable of offering both immune surveillance and proliferative capacity and consequently sustained protection (Bernasconi, Traggiai et al. 2002). Each population of immune cells has distinct cell surface markers and homing patterns (Sallusto, Lenig et al. 1999). Naive cells express both CD45RA and chemokine receptor CCR7. Central memory cells (T_{CM}) which typically home to lymph nodes express CCR7, but not CD45RA, and effector memory cells (T_{EM}) do not express CCR7. Absence of CCR7 expression allows migration to the site of infection (Sallusto, Lenig et al. 1999). Central memory cells are IL-2-secreting cells and this cytokine supports their expansion (Amyes, McMichael et al. 2005).

4.1.2 T cell helper function

CD4 T cells provide essential helper function for antigen-presenting cells such as B cells, monocytes and dendritic cells [DCs]. This is mediated in part through the interaction of the CD40 on antigen-presenting cells and CD154 (CD40-Ligand) on T cells (CD154 is expressed primarily on activated CD4 T cells), providing a signal for B cell clonal expansion and differentiation of Ig-secreting cells (van Kooten and Banchereau 2000; Ma and Clark 2009). For dendritic cells and monocytes/macrophages, this signal stimulates among other things the secretion of inflammatory cytokines including IL-12 that are central to the initiation of cell-mediated immune responses. The expression of CD154 is therefore central to the ability of CD4 T cells to provide appropriate 'help' for the development of cell-mediated immune responses and T cell dependent antibody responses. The critical role CD154 plays in controlling the fundamental aspects of the immune response has been demonstrated in individuals presenting with a genetic CD154 deficiency (the X-linked hyper-IgM syndrome). These individuals are susceptible to opportunistic infections and cancer (Levy, Espanol-Boren et al. 1997; Jain, Atkinson et al. 1999; Subauste, Wessendarp et al. 1999). Several groups have shown that the induction of CD154 in CD4+ T cells is decreased in HIV-1-infected persons (Zhang, Fichtenbaum et al. 2004; Subauste, Subauste et al. 2007).

In this study, we characterised naturally-acquired pneumococcal-specific T cell immunity (including T cell help) in individuals with asymptomatic HIV infection (WHO stage 1) and compared it to that of HIV-uninfected healthy controls. Functional properties of pneumococcal-specific T cells were characterised by measuring their proliferative capacity, their ability to produce cytokines (ELISpot and intracellular cytokine staining) and ability to provide T cell help (induction of CD154). Additionally, we compared the absolute numbers and/or relative proportions of different T cell subsets (naive T cells, central memory (T_{CM}), effector memory (T_{EM}), regulatory T cells (Tregs) and senescent T cells) in peripheral blood of healthy controls and HIV infected individuals. We tested the hypothesis that there is loss of function or specific subsets of CD4+ T cells that mediate protection against *S. pneumoniae* in HIV positive individuals, which could at least partially explain why this group is highly susceptible to invasive pneumococcal disease.

4.1.3 Study Rationale and aims

Protein antigen-specific immunity to the pneumococcus in the context of HIV has not been extensively studied. A better understanding of the influence of HIV infection on pneumococcal T cell immunity in the adult population can inform design and implementation of both antiretroviral therapy (ART) and protective vaccines. It is hypothesized that subtle functional defects in pneumococcal specific T cell immunity predispose those with HIV infection to IPD even before there is a numerical reduction in CD4 numbers. Therefore, the aim of this chapter was to investigate the effect of underlying HIV-1 infection on the function and phenotype of pneumococcal protein specific CD4+ T cells in Malawian adults (individuals with asymptomatic HIV infection [WHO stage 1]) even before the fall in CD4 count.

4.1.4 Study design

The study focused on a prospectively recruited otherwise healthy HIV-infected and uninfected adults. Adults were recruited from the voluntary counselling and testing clinic (VCT) or anti-retroviral (ARV) out-patient clinic at Queen Elizabeth Central hospital in Blantyre Malawi (as described Chapter 2: Materials and Methods, section 2.2 – 2.2.1). HIV status was tested and confirmed by two HIV rapid antibody tests Uni-gold (Trinity Biotech plc) and Determine (Abbott Laboratories). Subjects recruited were either healthy controls or asymptomatic and HIV infected (WHO stage 1). After informed consent, samples (venous blood and nasopharyngeal swabs) were collected from individuals fulfilled the inclusion criteria (Chapter 2: Materials and Methods, section 2.2 – 2.2.1). The collection of samples and the research complied with relevant guidelines and institutional practices of the College of Medicine (University of Malawi) and Liverpool School of Tropical Medicine (LSTM) research ethics committees (Protocols: P.03/08/626 and 08.41 respectively).

4.2 Materials and Methods

4.2.1 General materials and methods are as described in chapter 2.

Pneumococcal protein-specific T-cell immune responses were assessed using T cell CFSE proliferation assay, IFNγ T-cell ELISPOT assay, intracellular cytokine staining, cytokine ELISA, bioplex cytokine profiles in culture supernatants and expression of CD154 (Figure 4.1). Additionally, nasopharyngeal swabs were cultured to determine carriage rates. In this study we used whole pneumococcal protein antigens prepared from culture supernatants of a standard encapsulated type 2 strain (D39), a pneumolysin deficient mutant encapsulated type 2 (D39) isogenic pneumococci (Ply-), positive antigen control *M. tuberculosis* PPD and PHA as mitogenic control as described in Chapter 2 materials and methods (section 2.4). This work was done in collaboration with Dr Sarah Glennie and Mr David Mzinza as described in Chapter 2 materials and methods (sections 2.7 and 2.12).

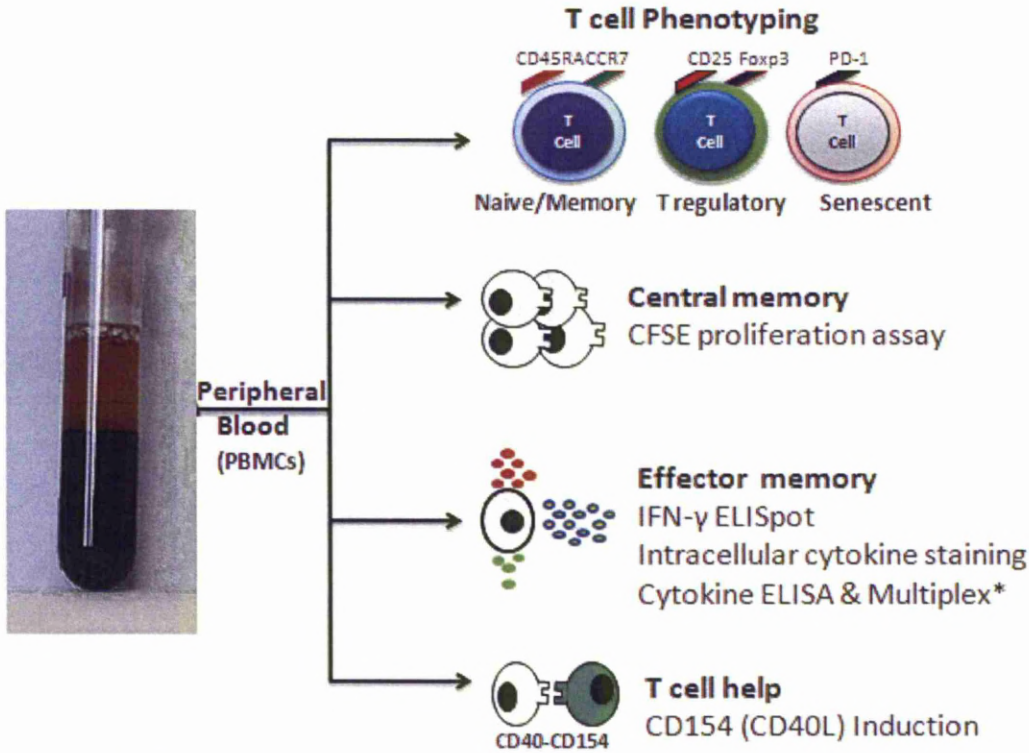


Figure 4.1 | Assays used to assess pneumococcal protein T cell immune responses.
* Bioplex cytokine profiles in culture supernatants- cytokines assessed come from T cells and other cell types. PBMCs- peripheral mononuclear cells.

4.3 Results

4.3.1 Demographic characteristics of the study population

A total of 53 asymptomatic WHO stage 1 HIV-infected adults (median age, 31 years [range, 18–58 years]) and 31 HIV-uninfected healthy adults (median age, 33 years [range, 23–53 years]) were recruited. The characteristics of the study cohort are shown in Table 4.1. Although, the HIV-infected subjects were asymptomatic and WHO stage 1, they presented with a wide range of CD4 T cell counts (median CD4 count, 252 cells/μl [range, 7-913 cells/μl]). The median CD4 T cell count of healthy controls was 853 cells/μl (range, 444–1323cells/μl). HIV-infected participants were classified on the basis of their $CD4^+$ T cell count (≤350,350-500 and ≥500 cells/μl) to allow assessment of carriage and immunity at different CD4 T cell counts. The CD4+ T cell count of 350 cells per microlitre was selected because it is the WHO recommended cut-off for initiating antiretroviral therapy while CD4+ T cell count of 500 cells per microlitre or more compares well with the CD4+ T cell counts of the majority of the HIV uninfected healthy adults recruited into the study. There was no difference in the nasopharyngeal carriage rate between the HIV-infected and HIV-uninfected. *S. pneumoniae* was detected in 14% of HIV-infected (≤350 cells/μl: -13 %, 350-500: 14% and ≥500: 13%) and in 16% of HIV-uninfected participants (P=0.2, by z test for proportions).

Table 4.1| Demographic characteristics of the study population

	HIV-uninfected	HIV-infected				P*value
		Overall	CD4>500$^{\beta}$	CD4 350-500$^{\beta}$	CD4≤350$^{\beta}$	
N	31	53	9	14	30	
Male (%)	68	54	13	50	76	
Female (%)	32	46	87	50	24	
Age, median yr (range)	33 (23-53)	31(18-58)	29(21-47)	28(23-40)	32(18-58)	
CD4, median (range)cells/μl	853(444-1323)	252(7-913)	695(549-913)	403(352-495)	192(7-292)	
Pneumococcal carriage (%)	16	14	13	14	13	0.2#

*HIV-uninfected versus HIV-infected (overall)
z test for proportions
β CD4 T cell counts (cells/μl)

4.3.2 Phenotypic assessment of T cells in HIV-uninfected and HIV-infected adults

CD4+ cell depletion is the hallmark of HIV infection (Brenchley, Schacker et al. 2004). However, observations based on total CD4+ cell counts alone may not accurately reflect the state of immunity in HIV infected individuals (Dunham, Cervasi et al. 2008; Prendergast, Prado et al. 2010). Therefore, a detailed assessment of immune cell subsets and the extent to which different subsets are impacted by HIV may not only be informative but accurately reflect the influence of HIV infection on T cell immunity or T cell responses to microbial antigens. To determine the changes that occur within the T cell subsets during the course of HIV infection the absolute numbers and/or relative proportions of CD4+ naive (T_N), central memory (T_{CM}) and effector memory (T_{EM}), T regulatory cells and senescent cells in peripheral blood of healthy controls and HIV infected individuals were assessed and compared.

4.3.2a Differential depletion of peripheral blood CD4+ T cell subsets in HIV-infected adults

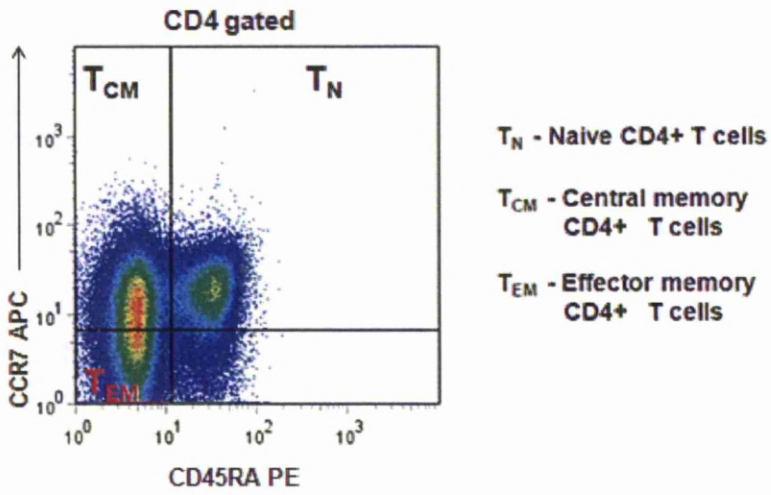
CD4+ T cell subsets were assessed by measuring the expression of CD45RA and CCR7 which characterises the state of differentiation (i.e. naive vs. central memory vs. effector) of CD4+ T cell subsets (Figure 4.2a). Analysis of the phenotype of the CD4+ T cell subsets revealed some differences between HIV-infected and uninfected persons. There was a significant decline in the proportion of T_{CM} compared to controls ($p=0.0043$, $U=115.5$) (Figure 4.2b) and a significant increase in the proportion of the T_{EM} subset in HIV-infected participants ($p=0.0226$, $U=141.5$) compared to HIV-uninfected individuals (Figure 4.2b). The proportion of the T_N subset in HIV-infected participants was comparable to that of HIV-uninfected individuals ($p=0.6501$, $U=224.0$) (Figure 4.2b).

In terms of absolute numbers of cells per cubic millimetre of blood, naive (T_N) and central memory (T_{CM}) CD4 T cell subsets declined significantly in HIV-infected individuals ($p=0.0009$; $U= 61.0$ and $p<0.0001$; $U= 18.0$ respectively), compared to HIV negative persons (Figure 4.2c). The absolute number of effector (T_{EM}) CD4+ T cell was lower but not significantly decreased ($p=0.0555$, $U=114.00$) compared to controls. These data show that the increase in the proportion of the T_{EM} subset observed in HIV positive persons is not due to an increased absolute number of the subset but rather to a selective loss of naive (T_N) and central (T_{CM}) CD4+ T cell subsets.

Additionally, these observations are in agreement with previous observations that HIV drives immune activation which results in a constant recruitment of CD4+ T cells from the naive and central memory pools into the effector pool (Hazenberg, Hamann et al. 2000; McCune 2001; Grossman, Meier-Schellersheim et al. 2002; Silvestri and Feinberg 2003) inevitably creating a strain on the homeostatic mechanisms of CD4+ T cell maintenance.

When the absolute counts were examined on the basis of CD4+ T cell count, we observed that there was a significant decline of the naive (T_N) and central (T_{CM}) CD4+ T cell subsets in HIV positive individuals with CD4+ T cell counts of 350-500 cell per microlitre (HIV uninfected vs. HIV-infected: T_N *p = 0.02 and T_{CM} *p=0.0003) while effector (T_{EM}) declined significantly in individuals with CD4+ T cell counts of $\leq$350 cells/ul (HIV uninfected vs. HIV-infected *p=0.003)(Figure 4.2d). The data indicate that effector memory CD4+ T cells decline at a slower rate compared with naive and central memory CD4+ T cell subsets.

a

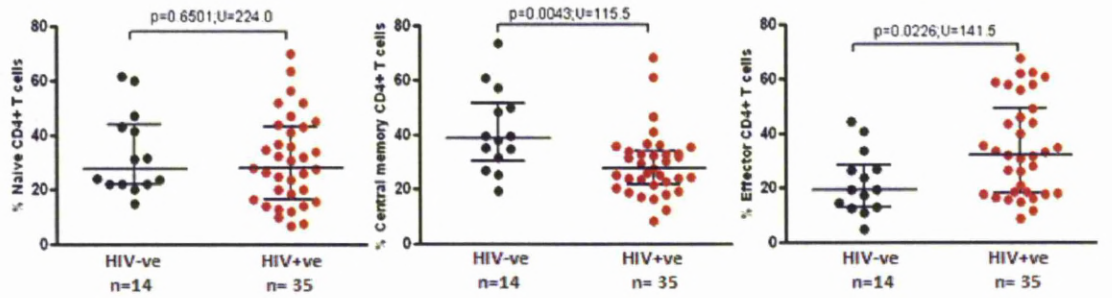


T_N - Naive CD4+ T cells

T_{CM} - Central memory CD4+ T cells

T_{EM} - Effector memory CD4+ T cells

b



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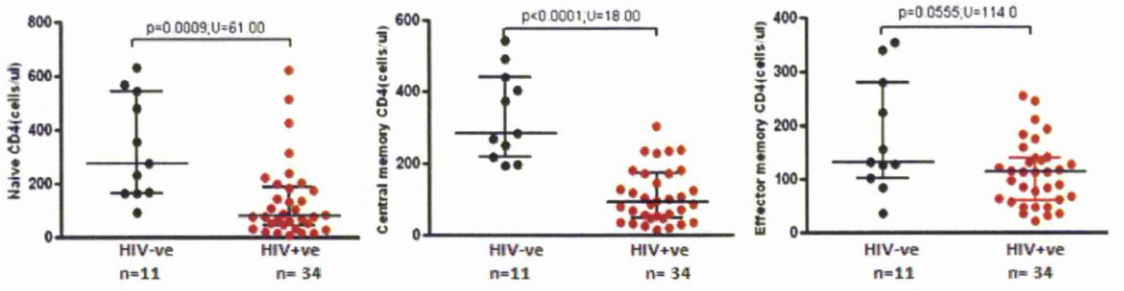


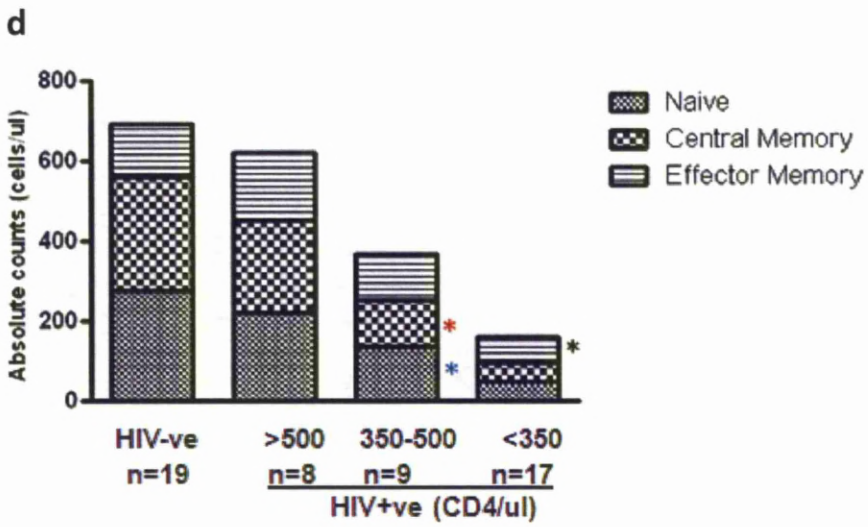


Figure 4.2 | Phenotype of CD4 T cells in PB of humans **a|**Representative flow cytometry dot plot showing naive and memory CD4 T-cell subsets gated on characteristic expression patterns of CD45RA and CCR7 (Upper left quadrant - central memory CD4+ T cells, lower left quadrant – effector memory CD4+ T cells and upper right quadrant – naive CD4+ T cells). **b|** Proportional representation of T_N, T_{CM} and T_{EM} in HIV-infected and HIV-uninfected adults **c|** Absolute numbers of peripheral blood CD4+ T_N, T_{CM} and T_{EM} in HIV-infected and HIV-uninfected adults and **d|** Absolute numbers of peripheral blood CD4+ T_N, T_{CM} and T_{EM} in HIV-uninfected and HIV-infected adults according to CD4+ T cell count (≥500, 350-500 and ≤350 cells/μl blood). HIV uninfected vs. HIV-infected: CD4 count 350-500 cells/ul T_N *p = 0.02, T_{CM} *p=0.0003 and CD4 count ≤350 T_{EM} *p=0.003).

4.3.2b Changes in regulatory T (Tregs) cells during HIV disease progression

Regulatory T (Tregs) cells have been implicated in the suppression of T cell activation and effector function (Weiss, Donkova-Petrini et al. 2004; Belkaid 2007; Keynan, Card et al. 2008). HIV- associated immune hyperactivation therefore may drive expansion and activation of Tregs population which may inhibit the development of effective immunity against microbes including *S. pneumoniae* leading to increased susceptibility to invasive pneumococcal disease. To assess this possibility, we compared the proportion and absolute number of T regulatory cells in peripheral blood of HIV-infected and uninfected adults. CD4+ Tregs cells were identified by $CD25^{hi}$ [top 15% CD25+ cells] and FoxP3 co-expression (Kinter, Horak et al. 2007) (Figure 4.3a). The frequency and absolute number of CD4+ $CD25^{hi}$FoxP3+T cells in the peripheral blood of HIV-positive adults were comparable to that of HIV uninfected individuals (p=0.6160, U=311.5 and p= 0.3525, U=100.0)(Figure 4.3b and c). Next, we assessed the relationship between the Tregs and CD4+ T cell counts, the latter being a predictor of HIV disease progression. There was a significant inverse correlation between absolute CD4 T cell count and Tregs (Figure 4.3d; p=0.0006, r = 06474.). The result is consistent with previous studies that have shown that HIV-infected individuals with low CD4+ T cell counts have, on average, an elevated percentage of Tregs compared to those with high CD4 level (Tsunemi, Iwasaki et al. 2005; Montes, Lewis et al. 2006; Cao, Jamieson et al. 2009).

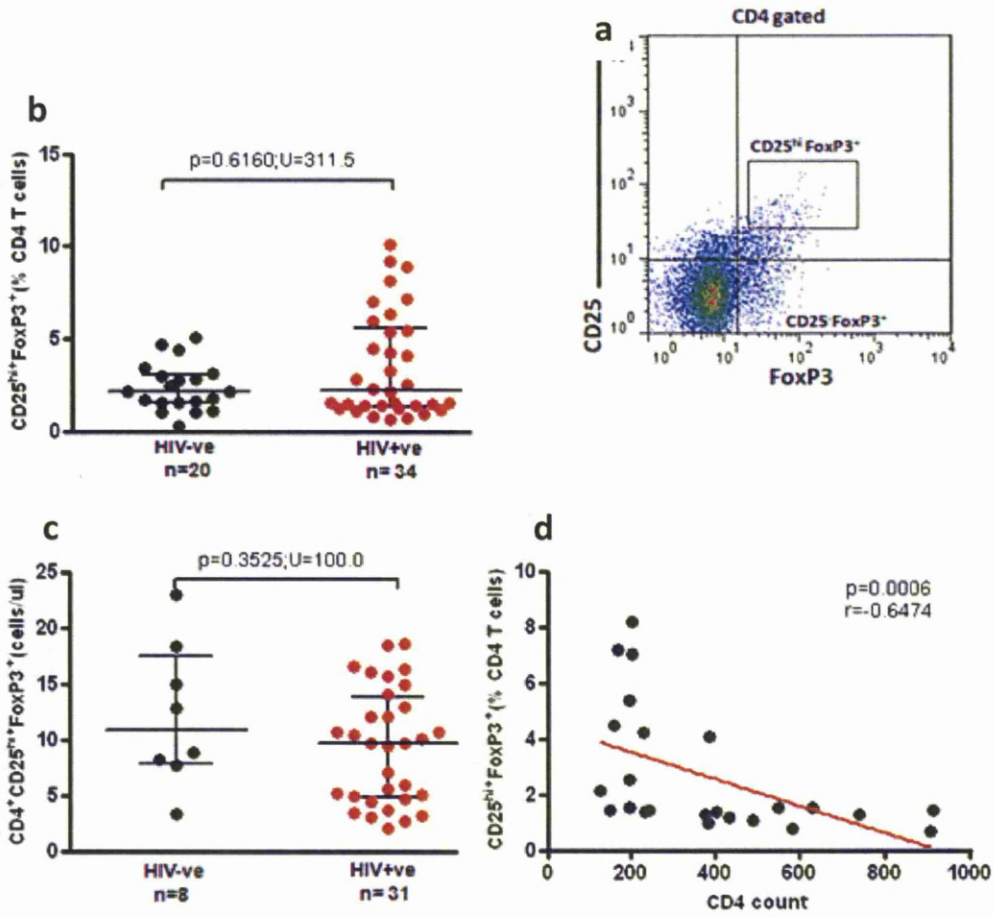


Figure 4.3|Phenotypic analysis of T regulatory cells in peripheral blood. a| Representative phenotypic analysis of CD25 and FoxP3 expression in peripheral blood CD4+ T cells from an HIV negative person **b|** The frequency of $CD25^{hi}$FoxP3+ Treg cells in HIV negative and infected persons **c|** Absolute numbers of CD4+ $CD25^{hi}$FoxP3+ Treg cells in PB of HIV negative and infected persons. **d|** Relationship between Tregs and CD4 T cell counts. Black horizontal bars represent median values of CD4 subsets. r-correlation coefficient. Differences were calculated using the Mann Whitney U test.

4.3.2c Relative expansion of CD4+PD-1+ senescent T cell in HIV-infected Adults

Previous studies have shown that programmed death-1 (PD-1) expression is upregulated on CD4+ T cells from people with chronic HIV infection, leading to decay of the CD4+ T cells and their function (Day, Kaufmann et al. 2006; D'Souza, Fontenot et al. 2007). This may have a negative impact on CD4+ T responses to microbial antigens including pneumococcal antigens, increasing susceptibility to microbial infections. PD-1 expression was higher in HIV-infected subjects than in HIV-negative subjects (Figure 4.4b, p=0.0003, U = 92.0). Assessment of the relationship between PD-1 expression and CD4 T cell counts, a predictor of HIV disease progression showed that there was no correlation between absolute CD4+ T cell count and PD-1 expression (p=0.1139, r = 0.2511). However, there was an elevated percentage of PD-1+CD4+ T cells in individuals with low CD4+ T cell counts (≤350 cells/ul) compared with those with high CD4 level (350-500 cells/ul)(Figure 4.4d; p=0.0100, U=27.0).

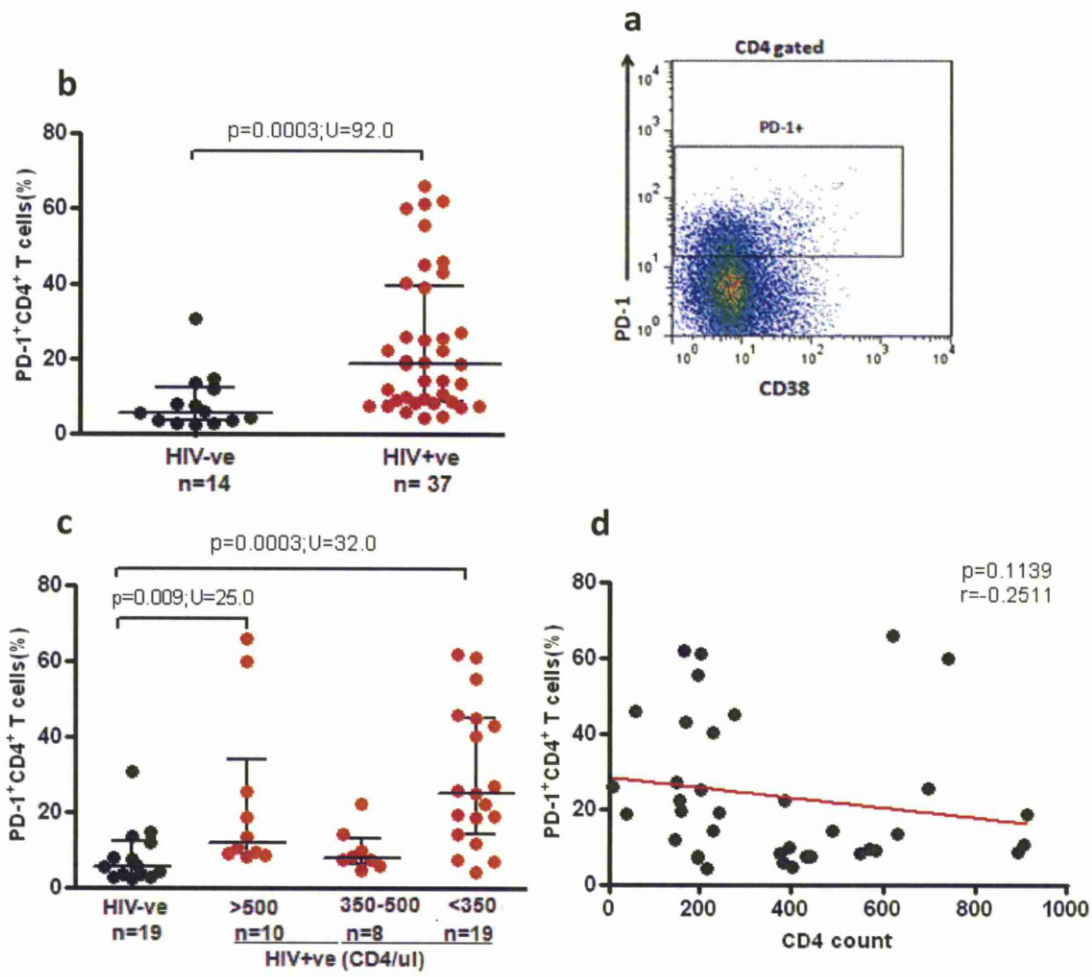


Figure 4.4 | PD-1 is upregulated on CD4+ T cell during HIV-infection. **a|** Representative plot PD-1 expression on peripheral blood CD4+ T cells (HIV-uninfected) **b|** Percentage of PD-1 expression on PB of HIV-uninfected and infected individuals **c|** Percentage of PD-1 expression on PB of HIV uninfected and infected individuals according to CD4+ T cell count **d|**Relationship between PD-1 expression and CD4 T cell counts. Black horizontal bars represent median values of CD4 subsets. r= correlation coefficient. Differences were calculated using the Mann Whitney U test.

4.3.3 Memory T cell responses to pneumococcal antigens

4.3.3a Impaired pneumococcal-specific CD4+ T cell proliferation

T cell proliferation has been shown to be important for T cell control of several diseases (Horton, Frank et al. 2006). Having established that circulating central memory T cells (T_{CM}) are depleted during HIV infection, we sought to establish whether the proliferative capacity of the remaining cells in response to pneumococcal antigens is compromised in HIV-infected individuals. PBMCs from healthy controls and HIV infected individuals were stimulated *in vitro* with pneumoCCS derived from either the WT pneumococcal strain (D39), Ply negative (-) D39 mutant, positive antigen control *M. tuberculosis* PPD and PHA as mitogenic control with unstimulated as background for 8 days, after which CD4 T cell proliferation was evaluated by CFSE dilution using flow cytometry (Figure 4.5).

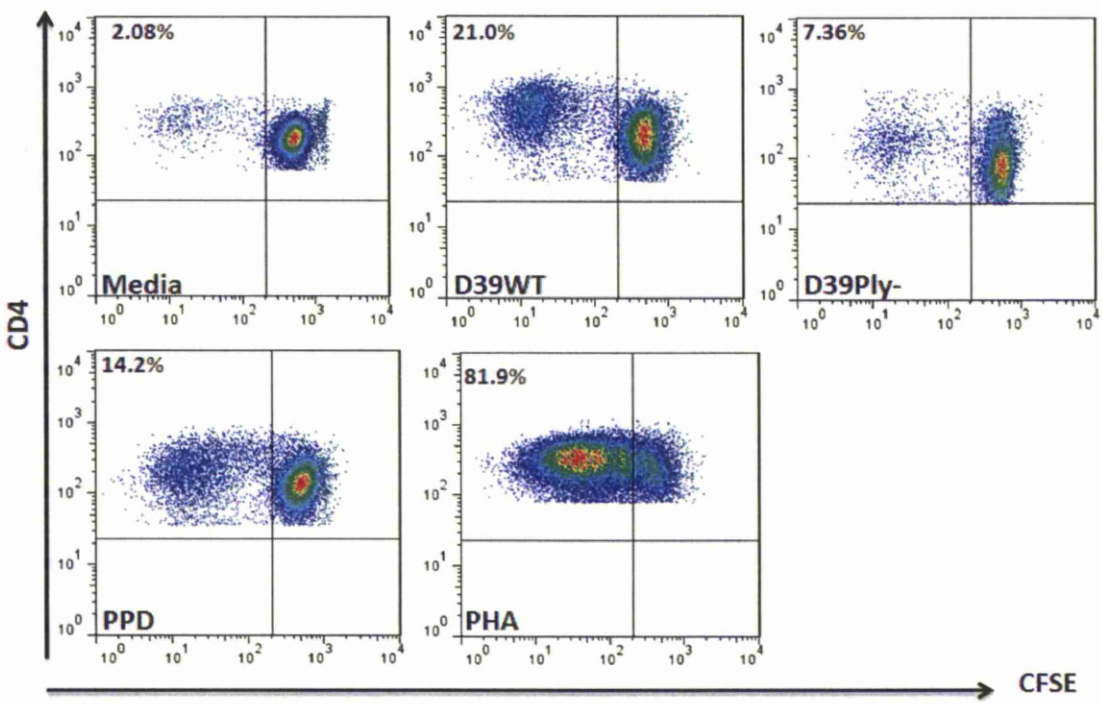


Figure 4.5 | Proliferative capacity of pneumococcal-specific CD4+ T cells. Representaive plots showing proliferation of PB CD4+ T cell toward pneumococcal and control antigens over a period of 8 days (HIV-uninfected). The percentage indicated in the upper-left quadrant represents CD4+ T cells that proliferated. Media- cells in RPMI 1640 supplemented with with penicillin/streptomycin, L-glutamine, HEPES and 2% heat-inactivated AB human serum.

The ability of CD4+ T cells from HIV-infected persons to proliferate in response to a mitogenic stimulus phytohemagglutinin (PHA) was comparable to that of HIV-uninfected individuals (HIV-uninfected-78%, HIV-infected-76%)(Figure 4.6d), indicating that HIV infection does not affect the intrinsic capacity of CD4+ T cells to proliferate. However, the capacity of CD4+ T cells to proliferate in response to pneumoCCS derived from a WT pneumococcal strain (D39), pneumoCCS derived from a Ply negative (-) D39 mutant and positive control antigen (PPD) was significantly impaired in HIV-infected individuals (Figures 4.6a, b and c). Since the proliferative responses to a mitogenic stimulus (PHA) were not impaired in HIV positive persons, impaired antigen specific proliferative responses suggests a defective antigen-CD4+ T cell interaction required to activate or maintain the central memory population.

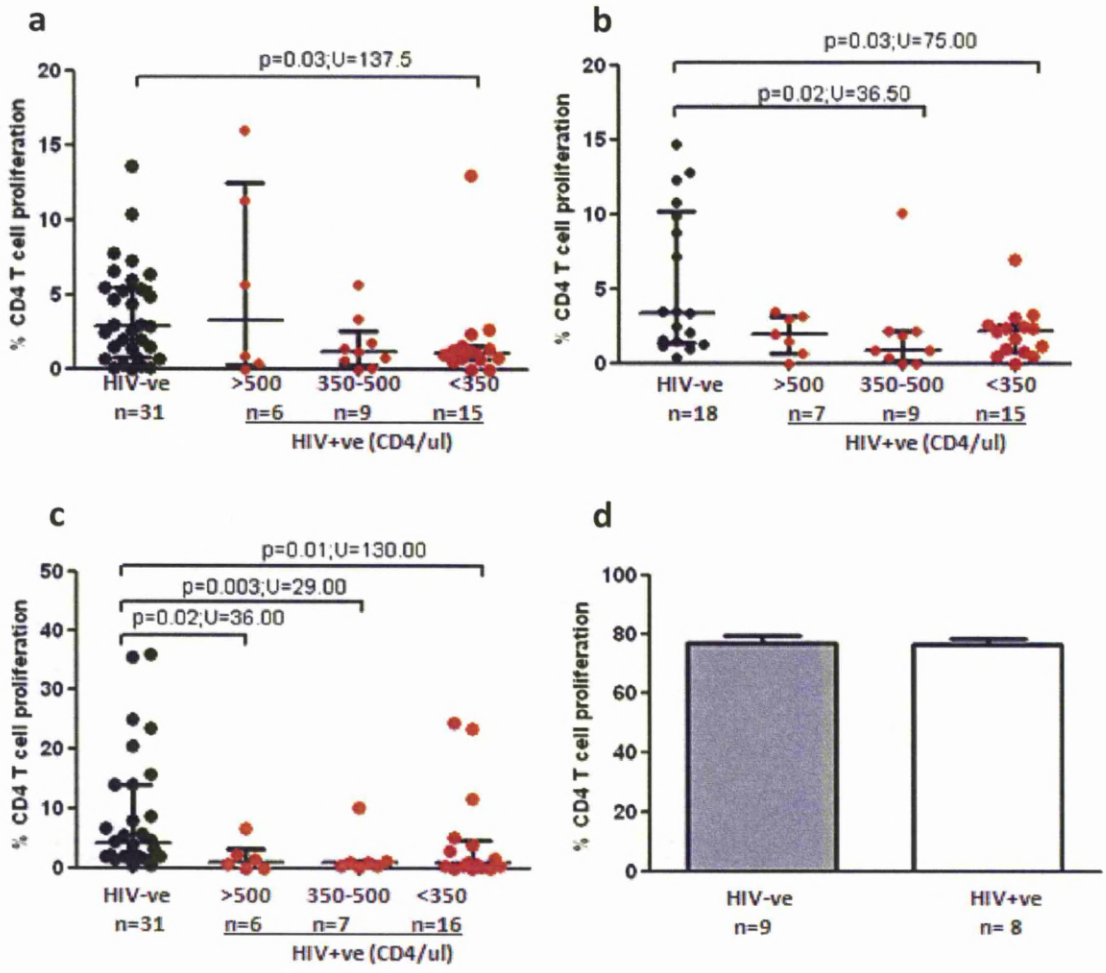


Figure 4.6 | Impaired proliferative capacity of pneumococcal-specific CD4+ T cells. Percentage of proliferating CD4+ T cells in response to **a|** pneumoCCS derived from a WT pneumococcal strain (D39) **b|** pneumoCCS derived from a Ply negative (-) D39 mutant **c|** positive antigen control M. Tuberculosis PPD. Black horizontal bars represent median values of total responses minus background. Differences were calculated using the Mann Whitney U test. **d|** mitogenic stimulus phytohemagglutinin (PHA). Graph represents median values of CD4 T cell proliferation minus background.

4.3.3b Reduced IFN-γ ELISPOT responses to pneumococcal protein antigens in HIV infected individuals

Work from this laboratory has demonstrated that pneumococcal protein antigens induce effector responses in the healthy adults as measured by an *ex vivo* ELISpot analysis (Mureithi, Finn et al. 2009). To examine the possibility that in addition to a total CD4 T-cell and T_{CM} deficit and impaired T_{CM} pneumococcal-specific proliferative capacity, the function of effector T cells is impaired in HIV-1–infected persons, pneumococcal effector memory (T_{EM}) IFN-γ responses were evaluated using a short-term ELISpot assay as described in chapter 2 materials and methods (section 2.7). PBMCs were stimulated for 16 hours with pneumoCCS derived from a WT pneumococcal strain (D39), pneumoCCS derived from a Ply negative (-) D39 mutant and a positive control antigen *M. tuberculosis* PPD before identifying the IFN-γ spot-forming cells by ELISpot assay.

Stimulation of PBMCs with WT pneumococcus generated a low number of effector memory IFN-γ producing cells and the responses were not affected by HIV infection (Figure 4.7a). *Ex vivo* IFN-γ ELISPOT responses to the pneumoCCS derived from a Ply negative (-) D39 mutant for HIV negative individuals, were not significantly different from responses induced by the pneumoCCS derived from aWT pneumococcal strain (p=0.0682, U= 40.0)(Figure 4.7d). However, the Ply negative responses were two times higher than those induced by the pneumoCCS derived from a WT pneumococcal strain (D39) (average 67.25 ± 61.82 spots/10^6 cells versus 31.42 ± 30.41spots/10^6 cells), suggesting an immunomodulatory effect of pneumolysin (Marriott, Mitchell et al. 2008). In addition, *ex vivo* IFN-γ ELISPOT responses to pneumoCCS derived from a Ply negative (-) D39 mutant decreased significantly in HIV positive persons (HIV-uninfected vs. HIV-infected CD4 count: ≥500: p=0.008, U=13.50; ≤ 350: p=0.02, U=39.0) (Figure 4.6b). E*x vivo* IFN-γ ELISPOT responses to PPD were reduced as well (HIV-uninfected vs. HIV-infected CD4 count ≥500: p=0.007; U=26.50) (Figure 4.7c).

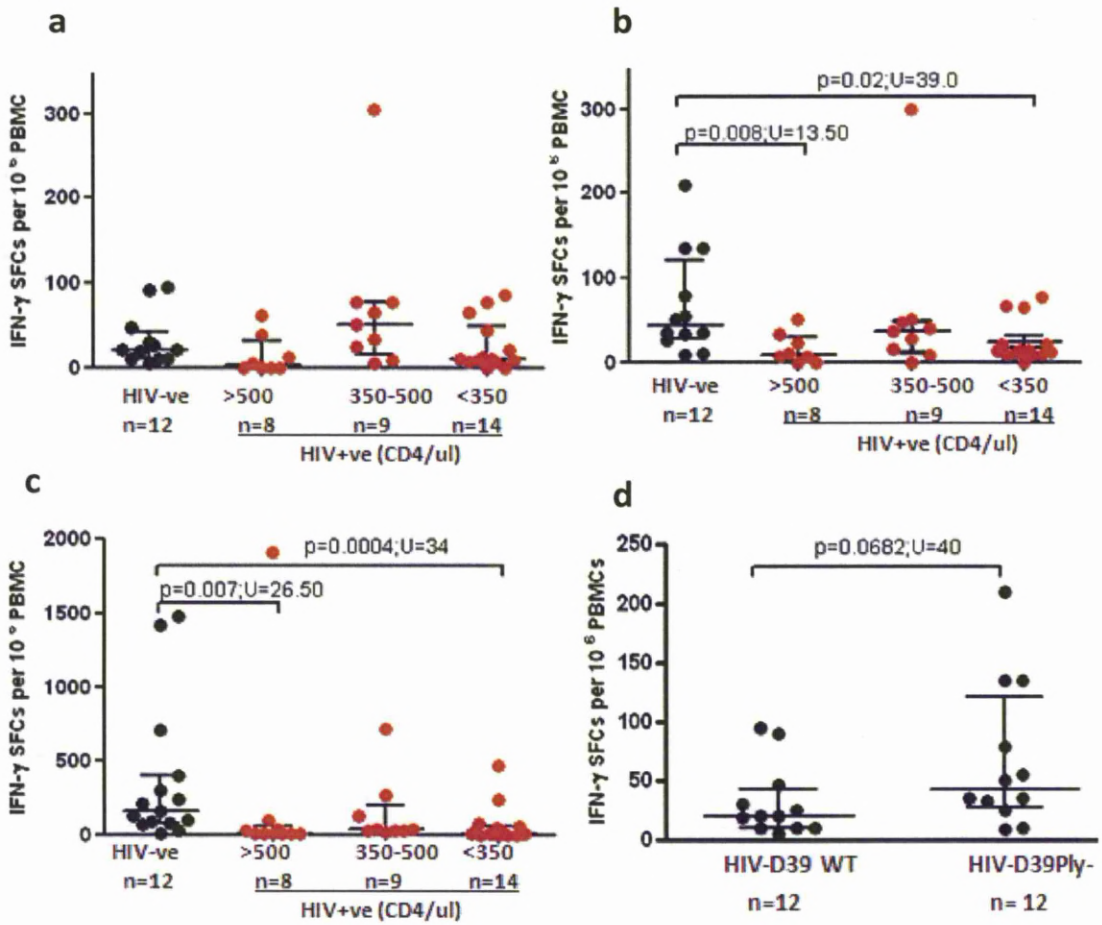


Figure 4.7 | *Ex vivo* interferon-g (IFN-g) ELISpot responses to pneumococcal protein antigens after overnight stimulation. IFN-γ ELispot response to **a|** pneumoCCS derived from a WT pneumococcal strain (D39) **b|** pneumoCCS derived from a Ply negative (-) D39 mutant **c|** positive control antigen M. tuberculosis PPD. **d|**Comparison between D39WT and D39Ply- induced responses in HIV negative individuals. Black horizontal bars represent median values and IQRs minus background responses. Differences were calculated using the Mann Whitney U test.

4.3.4 Defective induction of CD154(CD40L) on activated CD4 T cells

CD4+ T cells play a key role in the generation of immunity by providing appropriate 'help' for the development of humoral and cell-mediated immune responses. This pivotal role of CD154 (CD40L) in the regulation of numerous aspects of the immune response led us to examine whether the induction of this molecule in response to pneumococcal protein antigens is defective in HIV-1 infected persons. Impaired pneumococcal specific induction of CD154 in CD4+ T cells from HIV-infected individuals may contribute to poor T and B cell responses against *S. pneumoniae*. PBMCs from healthy controls and HIV infected individuals were stimulated *in vitro* with pneumoCCS derived from either the WT pneumococcal strain (D39), Ply negative (-) D39 mutant, a positive antigen control *M. Tuberculosis* PPD and PHA as a mitogenic control for 18 hours and CD154 expression evaluated on recently activated CD4+ T cells by flow cytometry (Figure 4.9a)

Activation of CD4+ T cells following stimulation was determined by the expression of the early activation marker CD69. The expression of CD69 after stimulation with pneumoCCS derived from a WT pneumococcal strain (D39) and pneumoCCS derived from a Ply negative (-) D39 mutant was similar between HIV infected and uninfected individuals (D39WT: p=0.0925, U=252.5 and D39Ply-: p=0.1024, U=287.5)(Figures 4.8a and b). Stimulation with PPD resulted in significantly lower expression of CD69 in HIV-infected persons compared to HIV negative persons (p=0.0056, U=216.5) (Figure 4.8c). This is consistent with the evidence from previous studies that have shown that for some pathogens HIV infection does not significantly change the expression of CD69 on CD4+ T cells following stimulation (Subauste CS, 2004) and that HIV infection does not alter the expression of the early activation marker CD69 (Card, McLaren et al. 2009). There were no differences between responses induced by the D39WT and D39Ply negative concentrated culture supernatants (p=0.8035, U=171.5)(Figure 4.8d).

The expression of CD154 on activated CD4+ T cells in response to pneumoCCS derived from a WT pneumococcal strain (D39) and pneumoCCS derived from a Ply negative (-) D39 mutant was impaired in groups of HIV infected individuals with CD4+ T cell counts of 350-500 cells/ul (D39WT:p=0.02; D39Ply-:p=0.03) and CD4+ T cell counts $\leq$ 350 cells/ul (D39WT: p=0.0001; D39Ply-:p=0.004)(Figures 4.9b and c).

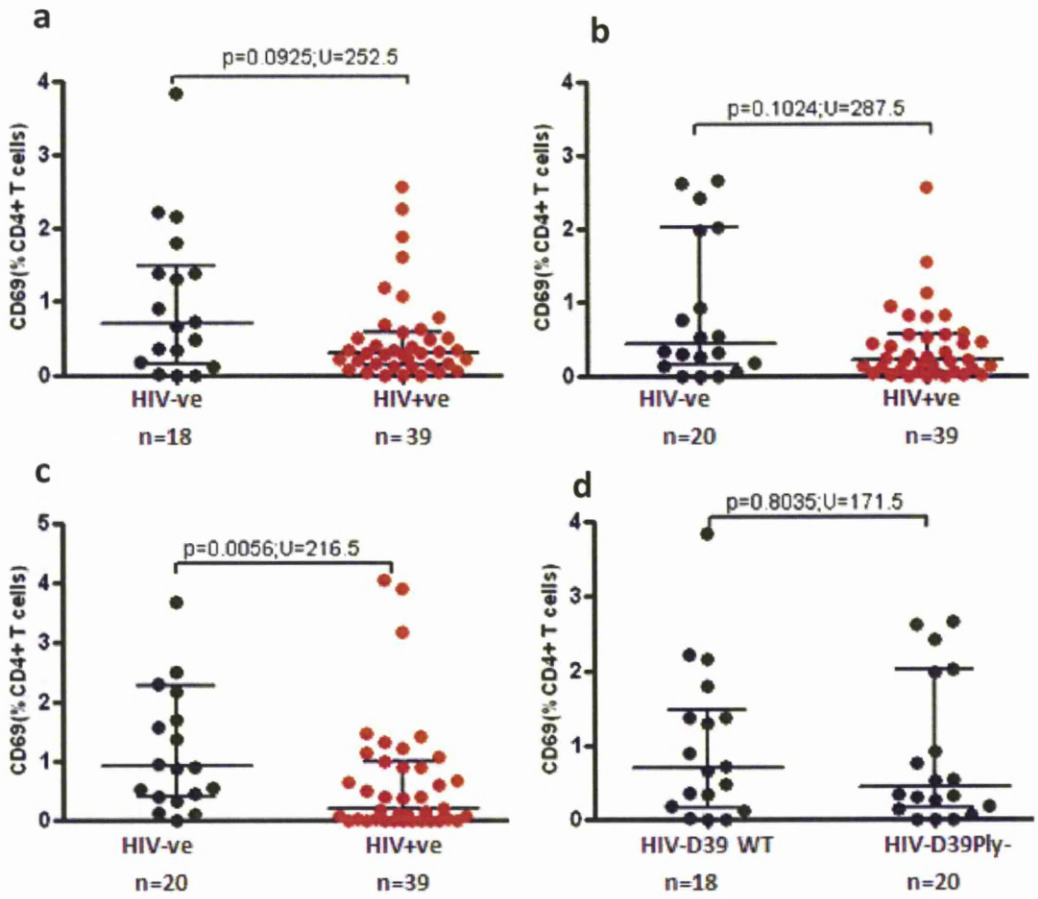
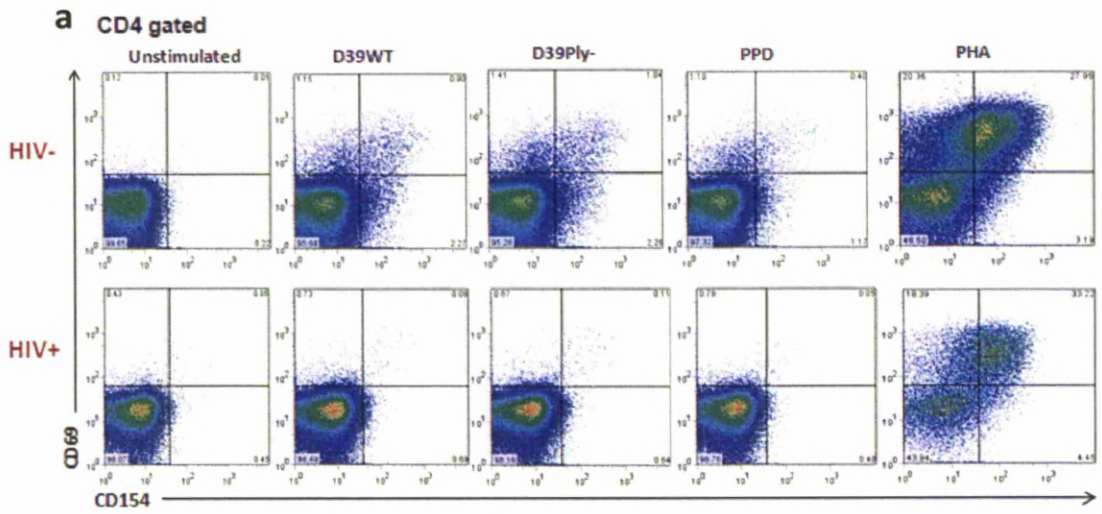
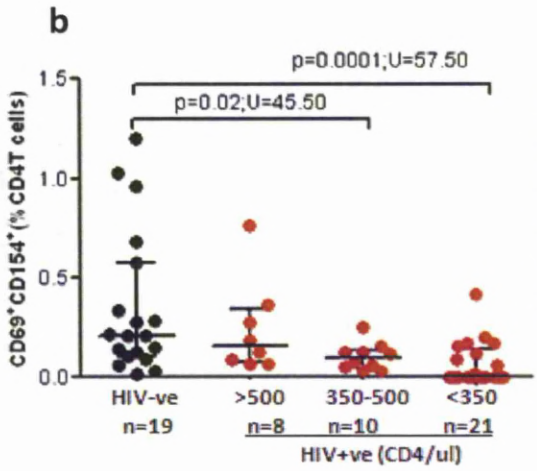
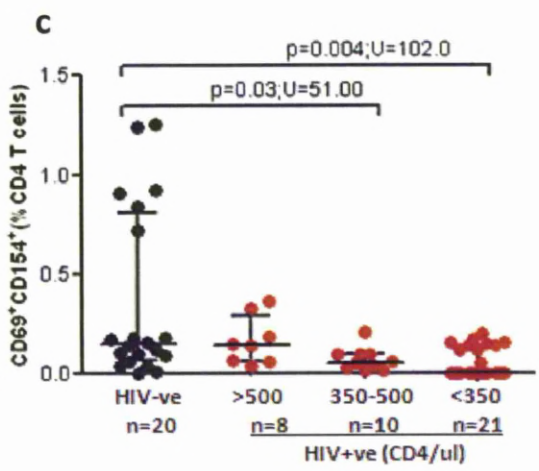


Figure 4.8 | CD69 expression in response to microbial antigens in CD4+ T cells of HIV-infected and uninfected. **a|** pneumoCCS derived from a WT pneumococcal strain (D39WT) **b|** pneumoCCS derived from a Ply negative (-) D39 mutant **c|** positive control antigen M. tuberculosis PPD **d|**Comparison between D39WT and D39Ply- induced responses in HIV negative individuals. Black horizontal bars represent median values of total responses minus background. Differences were calculated using the Mann Whitney

Induction of CD154 in response to PPD was defective in all 3 groups of HIV infected persons (CD4 count >500: p=0.04; 350-500: p=0.04 and ≤350: p=0.001) (Figure 4.9d). The intrinsic capacity of CD4 T cells to express CD154 was however not impaired as stimulation with mitogenic stimulus PHA showed no defect in CD154 expression in HIV infected individuals (Figure 4.9e).


a
CD4 gated
Unstimulated
D39WT
D39Ply-
PPD
PHA
HIV-
HIV+
CD69
CD154


b
p=0.0001;U=57.50
p=0.02;U=45.50
CD69+CD154+(% CD4T cells)
1.5
1.0
0.5
0.0
HIV-ve
n=19
>500
n=8
350-500
n=10
<350
n=21
HIV+ve (CD4/ul)


c
p=0.004;U=102.0
p=0.03;U=51.00
CD69+CD154+(% CD4 T cells)
1.5
1.0
0.5
0.0
HIV-ve
n=20
>500
n=8
350-500
n=10
<350
n=21
HIV+ve (CD4/ul)

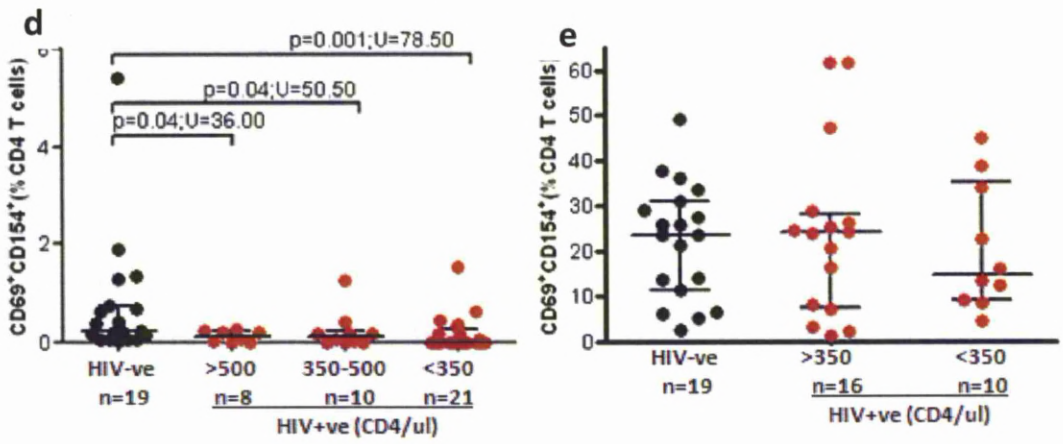


Figure 4.9 | Defective induction of CD154 (CD40L) on activated CD4 T cells from HIV+ persons. a| Representative plots showing expression of CD154 on activated CD4+ T cells from HIV-infected and uninfected individuals. **b|** Expression of CD154 in activated CD4+ T cells following stimulation with pneumoCCS derived from a WT pneumococcal strain (D39WT) **c|**CD154 expression following stimulation with pneumoCCS derived from a Ply negative (-) D39 mutant **d|** positive antigen control *M. tuberculosis* PPD and **e|** PHA. Black horizontal bars represent median values and IQRs of total responses minus background. Differences were calculated using the Mann Whitney U test.

4.3.5 Functional analysis of CD4 T cells (on the basis of their ability to secrete IL-2 and IFN-γ)

No single functional parameter can adequately quantify or identify the extent of an immune response. In addition to CD154, CD4 T cells are capable of expressing diverse cytokine profiles (De Rosa, 2004,). Therefore, we examined pneumococcal cytokine response, as defined by the number of CD4+ T cells expressing IFN-γ and IL-2 in HIV infected and uninfected individuals. Representative flow cytometry dot plots showing IL-2 and IFN-γ expression after pneumococcal antigen stimulation are shown in Figure 4.10a. No difference in the total cytokine response was seen between HIV infected and uninfected individuals in D39WT and D39Ply- stimulated PBMCs (Figures 4.10b & c). In HIV positive individuals, the D39WT and D39Ply-specific responses shifted from a predominantly IFN-γ response to an IL-2 dominated response (Figures 4.11a & b). PPD specific responses were dominated by IFN-γ regardless of HIV status (Figure 4.11c) and the CD4+ T cell response showed a distinct IFN-γ+IL-2+ double positive population (Figure 4.11c) which was not seen in pneumococcal-specific responses.

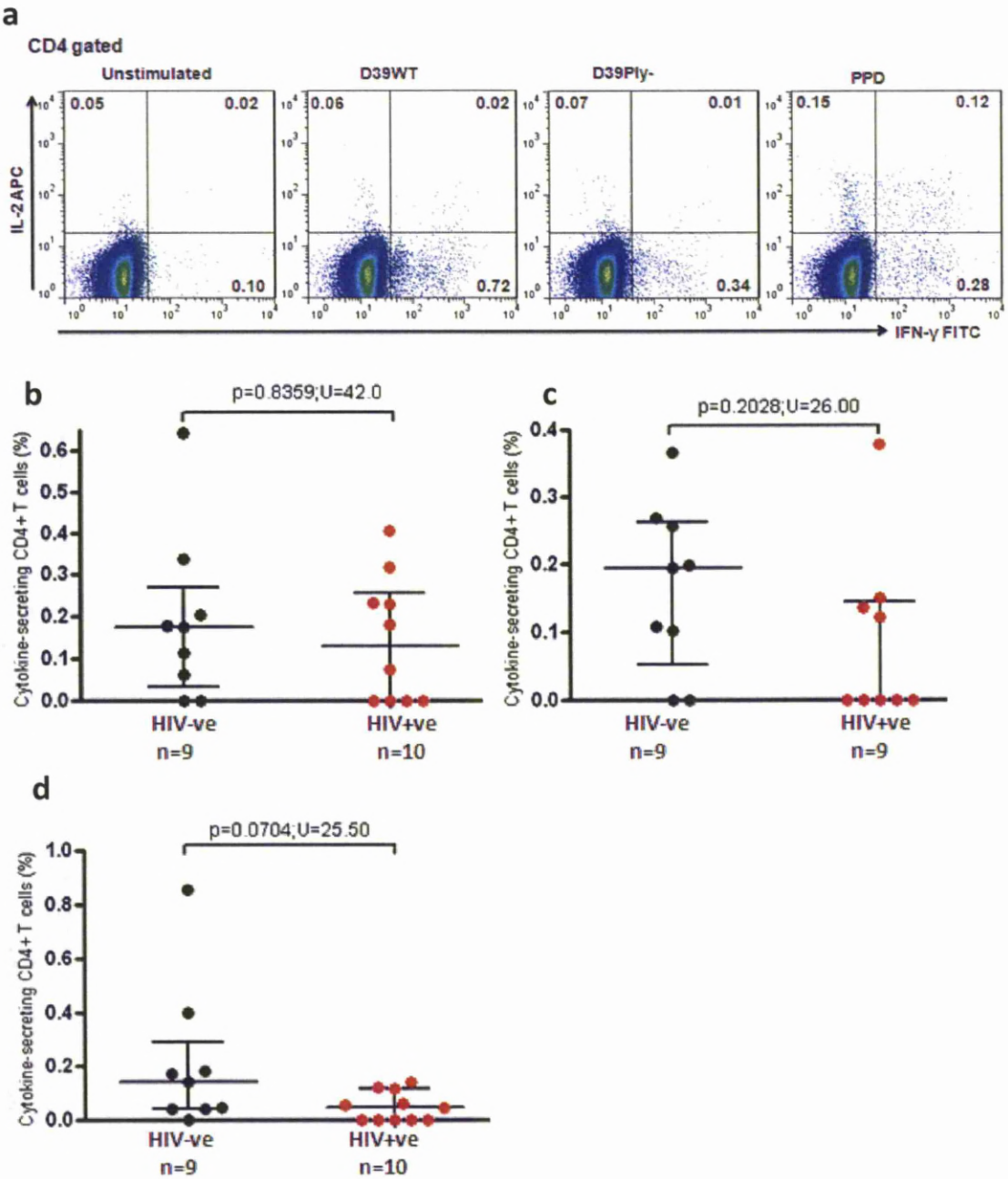


Figure 4.10 | Functional analysis of CD4 T cells. a| Representative flow cytometry dot plots showing IL-2 and IFN-γ expression after antigen stimulation of PBMCs from an HIV-negative person. Cytokine (CD4+ T cells expressing IFN-γ and IL-2) expression after stimulation of PBMCs from in HIV-uninfected (HIV-) and infected individual with **b|** pneumoCCS derived from a WT pneumococcal strain (D39WT) **c|**pneumoCCS derived from a Ply negative (-) D39 mutant **d|** *M. tuberculosis* PPD. The numbers in the quadrants represent the percentage of cytokine expressed. Differences were calculated

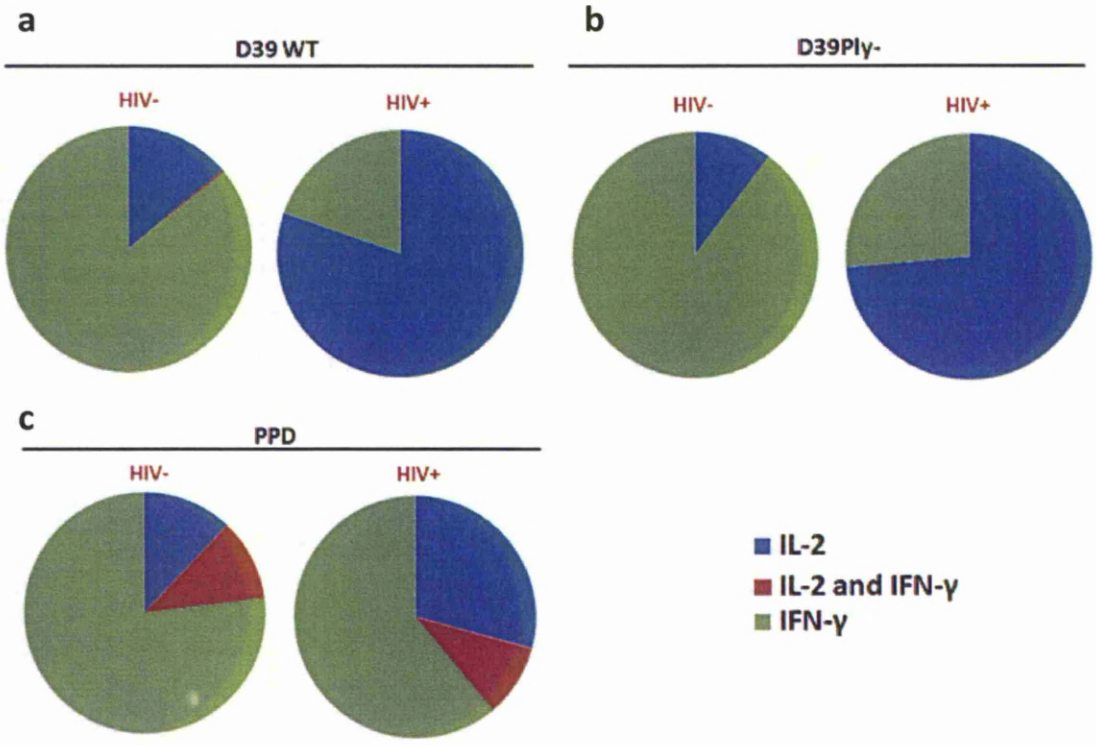


Figure 4.11 | Functionality of antigen-specific CD4+ T cells in PB. Distribution of **a|** WT pneumococcal strain (D39WT) **b|**pneumococcal Ply negative (-) D39 mutant **c|** *M. tuberculosis* PPD-specific cytokine responses in HIV-uninfected (n= 9) and HIV-infected (n=9-11). The pie charts represent mean frequencies of antigen-specific cytokine$^+$ cells,

4.3.6 Cytokine profile of the cellular immune responses to pneumococcal antigens

Work from this laboratory has shown that pneumoCCS derived from a WT pneumococcal strain (D39) and a Ply negative (-) D39 mutant induced IFN-γ, IL-10, IL-12(p40), IL-13, and IL-17 and TNF-α production quantified in culture supernatants obtained after 5 days of PBMC culture (Mureithi, Finn et al. 2009). IL-10 is a suppressive cytokine (Said, Dupuy et al.; Maynard, Harrington et al. 2007) while other cytokines have been shown to mediate clearance of pneumococcal colonisation (Lu, Gross et al. 2008; Zhang, Clarke et al. 2009) or control the multiplication of disseminated bacteria (Rubins and Pomeroy 1997; Sun, Salmon et al. 2007). We therefore investigated the dynamics of pro-inflammatory/regulatory responses to pneumococcal protein antigens during HIV- infection using cytokine IFN-γ and IL-10. Cytokines (IFN-γ and IL-10) were quantified in culture supernatants obtained after 7 days of PBMCs culture. The concentrations of the cytokine were determined on Bio-Plex 200 system. PBMCs from HIV-infected persons produced significantly less IFN-γ in response to pneumoCCS derived from a WT pneumococcal strain (D39WT) compared to controls ($p=0.007$) (Figure 4.12a). pneumoCCS derived from a Ply negative (-) D39 mutant ($p=0.07$). IL-10 production was also reduced in response to pneumoCCS derived from a WT pneumococcal strain (D39WT) ($p=0.003$)) (Figure 4.12b). There was a severe reduction in the concentration of IFN-γ compared to the relatively small change in IL-10 production (Figure 4.12c), which may create an environment that suppresses T cell immune responses to antigens.

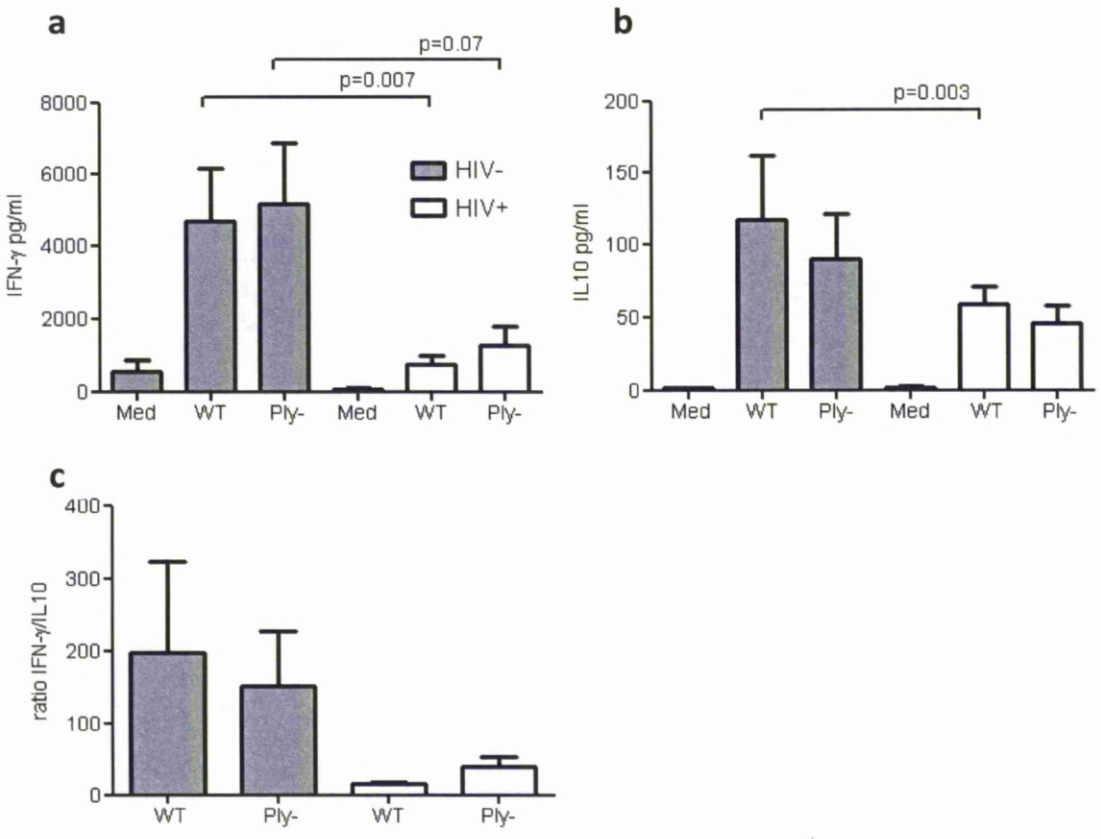


Figure 4.12 | Collated secreted cytokine profiles in PBMC culture supernatants stimulated with WT pneumococcal strain (D39), pneumococcal Ply negative (-) D39 mutant and medium (Med), as determined by bioplex cytokine assay. HIV-uninfected (grey)(n= 6) and HIV-infected (white)(n=9). Data are mean values standard error of the mean. Differences were calculated using the Mann Whitney U test. IFN-γ - interferon-γ.

4.4 Discussion

HIV-infected individuals are vulnerable to invasive pneumococcal disease and the role of protein antigen-specific immunity to the pneumococcus in this has not been extensively studied or is far from understood. In this chapter, we carried out a detailed assessment of CD4+ T cell subsets and characterised the functional properties of pneumococcal-specific CD4+ T cells in HIV infected and uninfected adults (in an area of relatively high HIV-infection and pneumococcal carriage rate). We show that in addition to the numeric loss of CD4+ T cell subsets during HIV infection, pneumococcal-specific CD4+ T cell responses are reduced or impaired. These observations may at least in part explain why HIV-infected individuals are at an increased risk of invasive pneumococcal disease.

Although other studies have shown an increase in pneumococcal carriage in HIV-infected adults (Rodriguez-Barradas, Tharapel et al. 1997; Madhi, Adrian et al. 2007; Gill, Mwanakasale et al. 2008), we found no increase of the nasopharyngeal carriage in our study population even in individuals with CD4 count less than 350 cells/ul (the WHO recommended cut-off for initiating therapy). HIV infection causes substantial depletion of CD4+ T cells in the gastrointestinal (GI) tract and vaginal mucosa (Brenchley, 2004, Guadalupe, M, 2003), however a similar depletion does not manifest at all mucosal sites, particularly the lung in human studies (Brenchley JM, 2008). Although it is still to be demonstrated, it is conceivable that the mucosal immunity (immunity in the upper respiratory tract) in our HIV positive participants is still relatively intact or robust enough to control pneumococcal colonisation (but not necessarily dissemination) and hence no change in the carriage rate. It follows then that the increased risk of invasive pneumococcal disease may be due to failure to control pneumococcal dissemination rather than colonisation.

In this study, we established that circulating central memory T cells (T_{CM}) are not only depleted during HIV infection but have a diminished capacity to proliferate in response to pneumococcal antigens (although not to mitogenic stimulus, PHA), implying that the antigen-T cell interaction required to activate and renew the central memory population is compromised.

T cell proliferation has been shown to be important for T cell control of several diseases (Horton, Frank et al. 2006) and therefore impaired proliferative capacity may have an effect on the ability of T cells to effectively control invasive pneumococcal infection. There was an elevated percentage of CD4+CD25^{hi}FoxP3+ regulatory T cells (Tregs) in HIV-infected persons with low CD4+ T cell counts. It is likely that overrepresentation of Tregs in this group may establish an increased suppressor-to helper ratio, which could lead to suppressed T cell immune responses to HIV and other pathogens including *S. pneumoniae*.

In mouse models of pneumococcal pneumonia and sepsis, IFN-γ has been shown to play a crucial role in mobilisation of the cellular immune responses to facilitate bacterial elimination from the tissues and from the bloodstream (Rubins and Pomeroy 1997; Sun, Salmon et al. 2007). In this study, the number of pneumococcal-specific T cells as determined by ELISpot was reduced during HIV infection, especially in the absence of pneumolysin, a potent TLR4 agonist and cognate antigen (Bernatoniene, Zhang et al. 2008). The proportion of CD4+ T cells producing IFN-γ in response to stimulation with pneumoCCS derived from a WT pneumococcal strain (D39WT), or pneumoCCS derived from a Ply negative (-) D39 mutant also declined. The amount of IFN-γ secreted into culture supernatants was decreased as well. It is therefore likely that impaired IFN-γ synthesis may lead to poor macrophage phagocytosis and microbial killing when recruited to the sites of infection and failure to control pneumococcal infection in HIV-infected persons. Severe reduction of IFN-γ in comparison to the relatively small change in IL-10 (a suppressive cytokine (Said, Dupuy et al.; Maynard, Harrington et al. 2007) secreted in culture supernatants may lead to suppressed immune response to pneumococcal antigens in HIV-infected persons. However, it is still to be demonstrated that IL-10 suppresses pneumococcal-specific responses.

In HIV positive individuals, pneumococcal-specific responses shifted from a predominantly IFN-γ response to an IL-2 dominated response. There are indications that IL-2 producing cells may be more susceptible to productive HIV infection than other cell types (Geldmacher, Ngwenyama et al. 2010). Therefore, a shift from cells producing predominatly IFN-γ to IL-2 producing cells may render the pmneumococcal-specific cells to HIV infection and depletion.

In this study, the induction of CD154 on activated CD4+ T cells in response to pneumococcal protein antigens was impaired during HIV-infection, even in individuals with CD4 cell counts above 350 cells/ul (presumably with relatively intact immunological features). This observation suggests a specific defect in the regulation of induction of CD154, above and beyond the loss of CD4+ T cells which is a hallmark of HIV infection. Patients presenting with genetic CD154 deficiency (the X-linked hyper-IgM syndrome) are susceptible to opportunistic infections and cancer (Levy, Espanol-Boren et al. 1997; Jain, Atkinson et al. 1999; Subauste, Wessendarp et al. 1999), and therefore it is likely that poor T cell CD154 expression in response to pneumococcal antigens may contribute to increased susceptibility to invasive pneumococcal disease.

There are multiple ways that defective CD154 signalling may increase susceptibility to infections including impaired humoral response, defective priming of T cells and poor macrophage activation. In mouse studies, CD154 has been shown to be essential for the antibody response to pneumococcal proteins and contributed to the humoral response to capsular polysaccharides and neutralization of CD154 suppressed the antibody response to PspA protein (Hwang, Nahm et al. 2000). Thus, impaired CD154 expression may affect antibody responses to pneumococcal proteins, contributing to poor control of IPD in HIV-infected persons.

In conclusion, this study provides evidence that reduced or impaired pneumococcal –specific CD4+ T cell responses may partly explain why HIV-infected are at increased risk of invasive pneumococcal disease. The study revealed considerable immune dysfunction in asymptomatic HIV infected Malawian adults. Pneumococcal specific memory T cells exhibited defective IFN-γ production, proliferation and CD154 expression. Additionally, there was an overrepresentation of regulatory T cells and a suppressive environment, because of a severe reduction in the concentration of IFN-γ compared to the relatively small change in IL-10 production. Together these observations demonstrate at least in part why HIV infection, with or without progression to acquired immunodeficiency syndrome (AIDS), dramatically increases the risk of pneumococcal invasive disease (IPD).

Given that pneumococcal immunity is susceptible to HIV-mediated immune disruption and that initiation of antiretroviral therapy leads to some functional immune reconstitution (Geng and Deeks 2009), the question therefore is: does pneumococcal specific immunity recover, or return, following initiation of antiretroviral therapy? This is the question the next chapter of this thesis sought to answer.

CHAPTER 5 PNEUMOCOCCAL IMMUNITY IN HIV-INFECTED ADULTS COMMENCING ON HAART – WHEN DOES IMMUNE RECONSTITUTION OCCUR?

5.1 Introduction

Evidence presented in chapter 4 together with work done by Dr Oluwadamilola H Iwajomo (PhD thesis 2011) in this laboratory showed that there is considerable compromise of pneumococcal specific T cell and B cell immunity in HIV infected Malawian adults. Initiation of antiretroviral therapy leads (although not completely) to CD4+ T cell recovery and functional immune reconstitution (Geng and Deeks 2009), coupled with a reduction in HIV-1 related opportunistic infections (Kaplan, Hanson et al. 2000), and a reduction in the incidence and magnitude of IPD (Grau, Pallares et al. 2005; Heffernan, Barrett et al. 2005; Klugman, Madhi et al. 2007). Questions remain, however, as to whether pneumococcal specific protective immunity returns or is re-constituted following initiation of therapy and how naturally-acquired pneumococcal immunity changes with ART immune reconstitution. Therefore, a study was designed to investigate the impact of ART on naturally-acquired immunity to pneumococcal protein antigens, mapping out the kinetics and the degree of immune reconstitution.

5.1.1 Immune Reconstitution during Highly Active Antiretroviral Therapy

5.1.1a Reconstitution of CD4 T cells

Initiation of ART leads to reconstitution of CD4+ T cells in the blood and mucosal sites (Kaplan, Hanson et al. 2000; Macal, Sankaran et al. 2008; Knox, Vinton et al. 2010). A longitudinal study of 23 HIV-infected persons on ART, showed that bronchoalveolar lavage (BAL) CD4 T cells (representing mucosal [pulmonary] CD4+ T cells), reconstituted after initiation of ART (Knox, Vinton et al. 2010). Based on Ki67 (a cell proliferation marker) expression by memory T cells, this reconstitution appeared to occur via local proliferation of resident BAL CD4+ T cells rather than redistribution. Reconstitution of CD4+ T cells in the gut-associated lymphoid tissue (GALT) has been shown to be slow and is substantially delayed compared to peripheral blood (Guadalupe, Reay et al. 2003).

Analysis of jejunal biopsy samples for Ki67 and integrin β_7 (which plays an important role in lymphocyte homing to the intestinal mucosa), indicated that intestinal CD4 T cell restoration following ART involved primarily CD4 T cell trafficking to the GALT from the periphery, and not local proliferation (Guadalupe, Reay et al. 2003). However, not all individuals on ART reconstitute CD4+ T cells at the same rate, or to the same extent (Gazzola, Tincati et al. 2009). As many as 30% of patients who are on ART present unsatisfactory CD4 T cell recovery despite suppression of HIV replication (Gazzola, Tincati et al. 2009; Kelley, Kitchen et al. 2009). Some of the most predictive factors for poor immune reconstitution are a lower CD4 nadir (Anthony, Yoder et al. 2003; Siddique, Hartman et al. 2006; McKinnon, Kimani et al. 2010; Negredo, Massanella et al. 2010), older age (Micheloud, Berenguer et al. 2008), increased immune activation (Hunt, Deeks et al. 2003; Benito, Lopez et al. 2005), altered T cell homeostasis (Benveniste, Flahault et al. 2005; Goicoechea, Smith et al. 2006), markers of microbial translocation (Jiang, Lederman et al. 2009) and HIV co-receptor usage (Delobel, Nugeyre et al. 2006).

Robbins *et al* observed that individuals with a lower baseline CD4 count at the time therapy is initiated achieved less immune reconstitution than those in the higher CD4 strata (Robbins, Spritzler et al. 2009). Additionally, it has been shown that initiation of therapy before CD4 T cell count falls below 350 cells/μl improves survival or better reconstitution of CD4 T cells (Guadalupe, Reay et al. 2003; Kitahata, Gange et al. 2009).

Several studies have demonstrated that antigen-specific CD4+ T cell responses recover following initiation of therapy (Lange, Valdez et al. 2002; Keane, Price et al. 2004; Furco, Carmagnat et al. 2008; Wilkinson, Seldon et al. 2009; Sutherland, Young et al. 2010). The degree of recovery is influenced at least in part by the prevalence of the antigen and baseline CD4+ T cell count. Studies have shown that there is either poor restoration of antigen-specific immune responses in individuals who are severely immunodeficient prior to treatment (Lange, Valdez et al. 2002; Sieg, Mitchem et al. 2002; Lederman, Williams et al. 2003; French, Keane et al. 2007) or the restoration is short-lived in this group (Keane, Price et al. 2004). Little is known however about the evolution of protein antigen-specific responses to the pneumococcus following initiation of ART.

5.1.1b Reconstitution of B cells

HIV infection leads to loss of circulating memory (CD19+CD27+) B-cell percentage and numbers in individuals with chronic HIV infection (De Milito, Morch et al. 2001; Nagase, Agematsu et al. 2001), and to memory B cell apoptosis (Titanji, Nilsson et al. 2003). Additionally, memory B cell responses and serological memory against both T cell dependent antigens (for example, influenza virus, measles virus and tetanus toxoid) and T-cell-independent antigens (pneumococcal polysaccharides) are decreased in HIV-infected individuals (De Milito, Nilsson et al. 2004; Malaspina, Moir et al. 2005; Titanji, De Milito et al. 2006; Hart, Steel et al. 2007; Tejiokem, Gouandjika et al. 2007). In a study of 28 patients with primary HIV infection and 40 with chronic HIV infection, there was a dramatic reduction in plasma levels of antibodies to measles and *S. pneumoniae* compared to controls and long-term non-progressors (Titanji, De Milito et al. 2006) and in a subset of this population (9 primary HIV infection[PHI] and 20 chronic HIV infection[CHI]), the number of measles-specific ASCs was significantly reduced in HIV-infected patients compared to controls. Additionally, the production of measles and pneumococcus-specific IgM and IgG antibodies in cell culture supernatants was impaired.

Studies show that the restoration of memory B cell numbers following initiation of therapy is slow and incomplete (De Milito, Morch et al. 2001; Chong, Ikematsu et al. 2004; D'Orsogna, Krueger et al. 2007; Moir, Malaspina et al. 2008), while restoration of B cell memory responses is either absent or incomplete (Malaspina, Moir et al. 2005; Bekker, Scherpbier et al. 2006; Titanji, De Milito et al. 2006; Hart, Steel et al. 2007). In a cross-sectional analysis of memory B cells in a cohort of 96 HIV-infected individuals De Milito *et al.* failed to detect any improvement in the proportion of memory B cells in patients undergoing antiretroviral therapy (De Milito, Morch et al. 2001). A study evaluating the effect of ART on serologic memory in patients with PHI treated for 6 months and in patients with CHI treated for 24 months, anti-measles titres did not recover and remained low compared with controls (Titanji, De Milito et al. 2006). In another study, initiation of therapy did lead to a reduction of spontaneous memory B cell apoptosis (Moir, Malaspina et al. 2008).

5.1.2 Study Rationale and aims

In the light of the findings presented in chapter 4 (and Dr Oluwadamilola H. Unuigbe-Iwajomo findings on B cell immunity, PhD thesis 2011), the literature showing variability in immune reconstitution following ART and the observation that adults and children on ART continue to have a higher incidence of invasive pneumococcal disease (IPD) than uninfected individuals, we hypothesized that the pneumococcal protein specific immunity may also be incomplete. This study aimed to provide a clearer understanding of the kinetics and the degree of reconstitution of pneumococcal immunity following initiation of ART in Malawian adults (over a period of 12 months). We also sought to determine whether therapy has any effect on nasopharyngeal carriage of *S. pneumoniae*, which provides an insight into immune reconstitution at the mucosal level and the impact of ART on herd immunity.

5.1.3 Study design

After informed consent, peripheral blood was collected from all individuals who fulfilled the inclusion criteria (chapter 2: section 2.2.3b). Additionally, nasopharyngeal swabs were collected and cultured for *S. pneumoniae* to monitor pneumococcal carriage. The participants were followed up for a period of 12 months during which samples were collected before initiation of therapy and at 3, 6 and 12 months ART. The data presented in this chapter however are for the first 6 months of ART (the final samples collection will be completed after submission of this thesis and will be collated with this data for publication). Samples were also collected from HIV-uninfected healthy controls at month six (6) of the study (Chapter 2: section 2.2.3a) to assess the degree of reconstitution at 6 months ART. In line with previously published reports (Wilkinson, Seldon et al. 2009), controls used in this study were HIV-uninfected individuals and therefore not on ART. Although the perfect controls for the study would have been HIV-uninfected individuals given antiretroviral drugs, there is no published evidence that antiretroviral drugs have sufficient effects on T or B cell function directly, to account for the differences seen. In addition, in view of the numerous haematological, neurological and metabolic side effects of the drugs it would have been ethically unacceptable to administer ART to otherwise healthy over even a short time period.The immune responses obtained from controls used in the study, serve as a 'goal post' or an indicator of where the immune responses ought to be if immune reconstitution is complete.

As described in section 2.2.3 (materials and methods chapter), this study was approved by the College of Medicine (University of Malawi) and Liverpool School of Tropical Medicine (LSTM) research ethics committees (Protocols: P.07/09/801 and 09.71 respectively).

5.2 Materials and Methods

5.2.1 General materials and methods are as described in chapter 2.

Antigen specific T and B cell immune responses were assessed using T cell proliferation assay, T (IFN-γ) and B cell ELISpot assays, intracellular cytokine staining and expression of CD154 (Figure 5.1). Peripheral blood T and B cell immunophenotypes were evaluated as described in chapter 2 materials and methods (section 2.5). Additionally, nasopharyngeal swabs were cultured to determine carriage rates.

In this study we used whole pneumococcal protein antigens prepared from culture supernatants of a standard encapsulated type 2 strain (D39), a pneumolysin deficient mutant encapsulated type 2 (D39) isogenic pneumococci (Ply-), positive antigens control *M. tuberculosis* PPD and Inactivated influenza vaccine (Split Virion) BP (2009/2010 vaccine (Chapter 2: materials and methods; section 2.4) for T cell assays. To assess B cell immune responses we used pneumococcal recombinant proteins: CbpA, PspA, PsaA, and a combination of tetanus toxoid, PPD and Flu as a positive control (Chapter 2 materials and methods [section 2.4]). This work was done with the assistance of Mr David Mzinza.

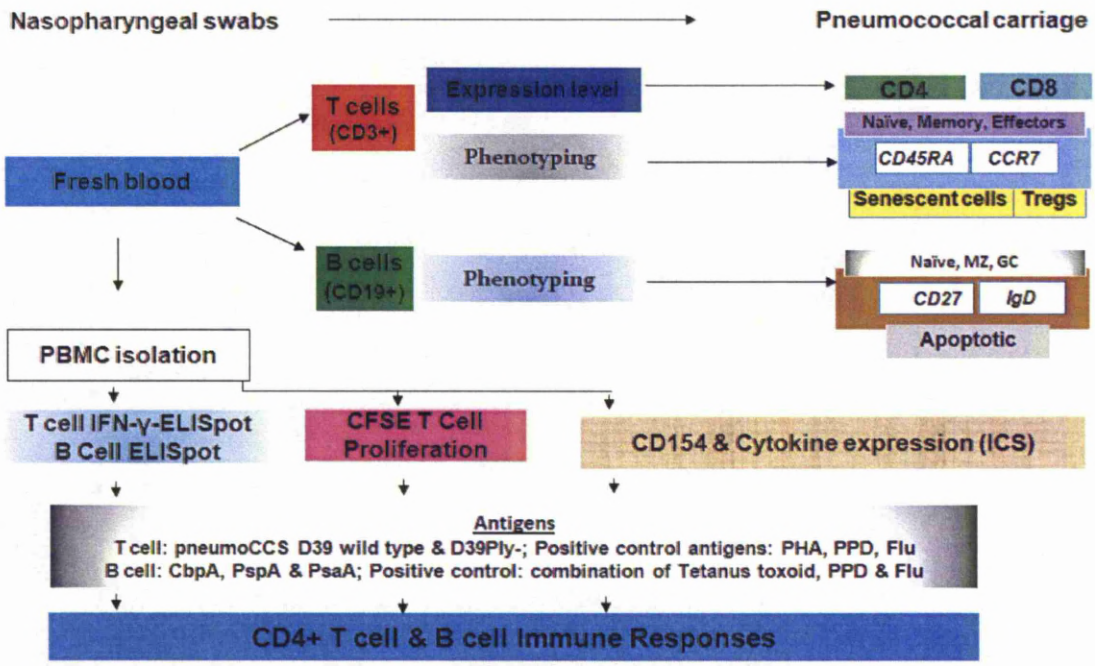


Figure 5.1 | Flow chart for immunonological analysis. T cell immune responses were assessed by T cell proliferation, IFNγ T cell ELISpot, intracellular cytokine staining assays and expression of CD154 using pneumoCCS D39WT and D39Ply- and positive antigen controls *M. tuberculosis* PPD and Flu. B cell immune responses assessed by B cell ELISpot assay using recombinant proteins: CbpA, PspA, PsaA, PdB and a combination of tetanus toxoid, PPD and Flu as a positive control.

5.3 Results

5.3.1: Characteristics of the HIV-1 infected subjects commencing on ART

A total of forty eight HIV-positive patients initiating ART were enrolled to the study (median age 37; range of 25-55 years: 21 (44%) males and 27 (56%) females). 46 patients were initiated on Triomune (stavudine, lamivudine and nevirapine), one patients received stavudine, lamivudine and efavirenz while the other received Zedolum N (zidovudine, lamivudine and nevirapine). During the follow up period (6 months ART), 2 participants died and 4 were lost to follow up. The median nadir absolute CD4+ T cell count was 159 cells/µl (range 3-573 cells/µl) increasing to 249 cells/µl (range: 63-760) at 6 months of antiretroviral therapy (Table 5.1) (see section 5.3.2). Baseline CD4+ cell counts were as follows: 15 patients had CD4+ cell counts ≤100 cells/µl; 18 had CD4+ cell counts between 101 and 200 cells/µl, 8 had CD4 cell counts between 201 and 350 cells/µl and 6 had CD4+ cell counts >350 cells/µl (commencement of ART was based on WHO clinical staging as per national guidelines as opposed to CD4 counts).

As shown in table 5.1 at the start of the study, *S. pneumoniae* was detected in 27% (13/48) of study participants which increased to 33% (14/43) at 3 months on ART and to 40% (17/40) at 6 months on ART. The carriages rates were not statistically different (p=0.189, Fischer's exact). To assess whether there is viral suppression in the study participants, plasma viral load was determined for 27 participants (27/43) at 6 months ART. All participants had viral load less than 5000 copies/ml. 11 had undetectable viral load, 9 had 100 - 1000 copies/ml, 4 had 1000 -2000 copies/ml and 2 had viral load between 2000 and 4000 copies/ml.

Table 5.1 | Baseline characteristics of the HIV-1 infected subjects commencing on ART

	Time on ART			
	Baseline	**3 months**	**6 months**	***P****
No. Subjects	48	44	43	
Male [No.%]	21 (44)	NA	NA	
Female [No. %]	27(56)	NA	NA	
Mean age [years(range)]	37(25-55)	NA	NA	
CD4+ counts				
median (range), cells/μl	159(3-573)*	264(17-737)*	249(63 - 760)	<0.0001*
median Δ, cells/μl	NA	111.5(-50 -378)	114(-178 - 335)[¶]	
S. pneumoniae carriage [No. %]	13/48 (27)[#]	14/43(33)	17/42(40)[#]	0.189[#]

[¶]Baseline versus 6 mths on antiretroviral therapy (ART), # baseline versus 6 mths on ART, * # baseline versus 3 mths on ART

5.3.2 Phenotypic Analysis of T Cells

5.3.2a Recovery of CD4 T cell counts in adults during HAART

To examine immune recovery upon initiation of therapy, CD4 counts were measured in HIV-infected patients at months 0 (baseline/pre-therapy), 3 and 6 on antiretroviral therapy (ART). For comparison, measurements of CD4 counts were done in controls at the time of the 6 months sampling of the ART patients. All HIV-infected subjects showed evidence of a CD4+ T cell increase after initiation of antiretroviral therapy. The CD4+ T cell count increased rapidly during the first 3 months (Figure 5.2; $p<0.0001$) and then remained relatively unchanged for the next 3 months. The median change from baseline was 111.5 cells/µl at 3 months ART (Table 5.1). This is consistent with previous reports which showed a biphasic reconstitution of CD4+ T cells: a steep increase during the first 8-12 weeks followed by a more gradual increase of CD4+ T cells (Lederman 2001; Gandhi, Spritzler et al. 2006; Robbins, Spritzler et al. 2009). CD4+ cell counts of the HIV infected were still significantly lower at 6 months ART compared to the controls (Figure 5.2; $p=0.0001$, $U=29.0$).

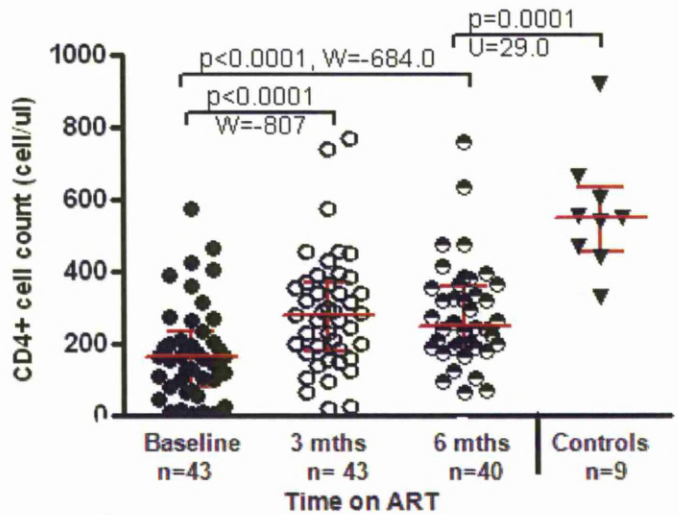


Figure 5.2| Recovery of peripheral blood CD4 T cell counts in adults on antiretroviral therapy a| CD4 T cell counts were measured at baseline (pre-therapy), 3, 6 months ART and in controls at month 6 of the study. Red horizontal bars represent median values of CD4 subsets. Statistical significance was analysed using the Wilcoxon matched pairs when comparing CD4+ T cell counts of HIV-infected persons on ART, and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison.

5.3.2b CD4+ naive and memory cells in adults during HAART

Naive, memory and effector T cell subsets were assessed based on the expression of CD45RA and CCR7 (as described in Chapter 4 section 4.3.2). Naive T cells (T_N) were defined as CD45RA+CCR7+, central memory (T_{CM}) as CD45RA-CCR7+ and effector memory (T_{EM}) as CD45+CCR7-. Consistent with the data shown in chapter 4 (section 4.3.2a), we found that the relative size of the population of naive CD4+ T cells is not affected by HIV infection (Figure 5.3a). There were no changes in the proportion of central and effector memory T cells in HIV positive participants over the six months of ART (Figures 5.3c and e). The proportion of central memory T cell remained significantly lower compared to controls at 6 months ART (Figure 5.3c, $p<0.0001$) while the effector memory cells remained significantly elevated at 6 months ART (Figure 5.3e, $p=0.0087$, $U=99.00$). As expected there was an increase in the absolute counts of naive, central and T_{EM} over the six months of ART (Figures 5.3 b, d and f; T_N: $p=0.0007$; T_{CM}: $p=0.0002$; T_{EM}: $p=0.0003$ at 3 months ART) but far from achieving the levels seen in the controls at 6 months ART ($p=0.0413$, $U=76.00$; $p<0.0001$, $U=8.00$; $p=0.0105$, $U=60.00$ respectively).

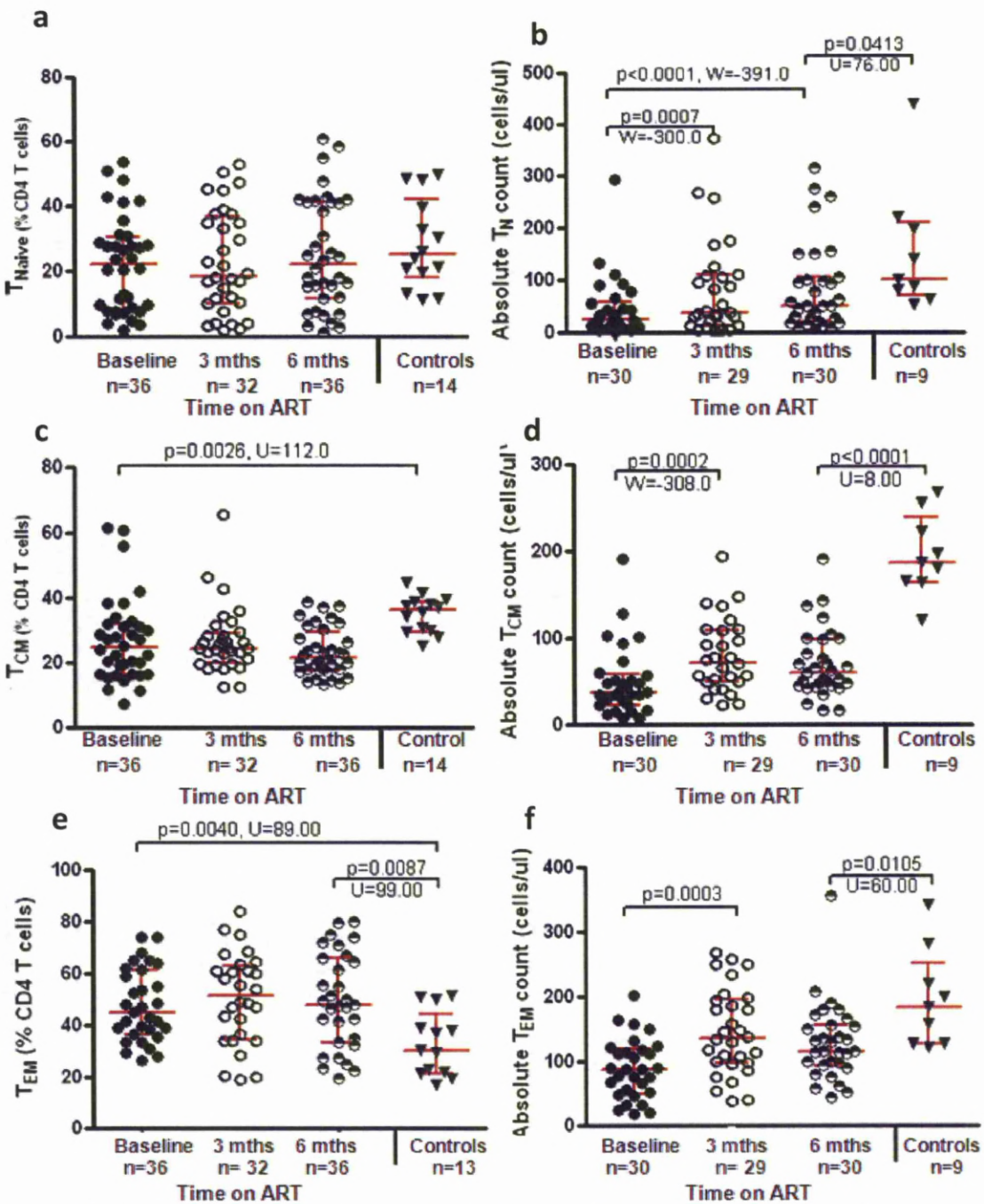


Figure 5.3|Incomplete reconstitution of CD4+ T subsets in peripheral blood of adults on antiretroviral therapy a, c and e| The frequency of naive CD4+ T cells (T_N: CD45RA+CCR7+), central memory CD4+ T cells (T_{CM}: CD45RA-CCR7+) and effector memory CD4+ T cells (T_{EM}: CD45RA-CCR7-) in PB of HIV negative and infected persons on ART **b, d and f|** The frequency of T_N T_{CM} and T_{EM} CD4+ T cells in PB of HIV negative and infected persons on ART. Red horizontal bars represent median values. Differences were calculated using the Wilcoxon matched pairs (HIV+ versus HIV+) and the Mann Whitney U tests (HIV+vs HIV-).

5.3.2c Changes in Tregs and Senescent CD4+ T cells during HAART

In chapter 4, we showed that Tregs expand as HIV disease progresses and therefore we sought to determine whether Tregs level changes during therapy. To determine Treg levels in HIV-infected patients on ART, CD4+CD25high+FoxP3 cells (as percentages of CD4+ cells) were measured at baseline, month 3, and month 6. Controls were assessed at month 6 of the study. At baseline, median percentage of Tregs in HIV-infected patients was 2·45% (0·95–7·5%) of CD4+ cells. The median percentages decreased following initiation of therapy (1.13 [0.30-3.73] at 3 months ART, p<0.0001(W=403.0) and 1.32% [0.61-4.65%] at 6 months ART, p<0.0001). The median percentage of Tregs of the patients was still significantly higher compared to the controls [0·65% (0.27–1·47%), p=0.0002, U=51.00] at 6 months ART (Figure 5.4).

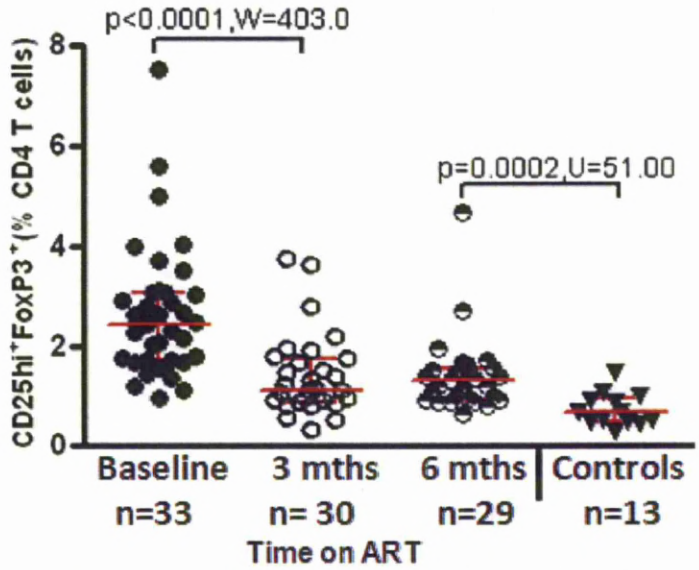


Figure 5.4|Phenotypic analysis of T regulatory cells in peripheral blood of adults on antiretroviral therapy. a| The frequency of CD4+CD25^{hi}FoxP3+ Tregs cells in HIV negative (controls) and infected persons on ART at baseline, month 3 and 6. Red horizontal bars represent median values of CD4 subsets. Statistical significance was analysed using the Wilcoxon matched pairs when comparing Tregs of HIV-infected persons on ART, and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison.

Chronic immune activation and persistent infections in HIV-1 infected individuals provide an environment that favours accelerated replicative senescence of T cells (Cao, Jamieson et al. 2009; Desai and Landay 2010). One characteristic feature of senescent T cells is the loss of CD28 costimulatory molecule (Merino, Martinez-Gonzalez et al. 1998) and increase in the proportion of CD57+ T cells (Brenchley, Karandikar et al. 2003). These markers were used to assess the effect of antiretroviral therapy on T cell aging. Whole blood was incubated with anti-human antibodies CD3/CD4/CD28/CD57 and analysed for replicative senescence by determining the frequency of CD4+CD28-CD57+ T cells (Figure 5.5a).

In patients, median percentages of CD4+ CD28-CD57+ decreased after initiation of ART (Figure 5.5b; baseline: 1.87% [0·17–59·34%], 3 month ART: 1·02% [0·02 – 26·26%, p=0.0005, W=407.0] and 6 month ART: 1.21% [0·08– 36·91%]). The median percentage of senescent cells in controls was 0.42% (0.12-2.85%) and significantly lower compared to baseline (p = 0·0242, U=89.0) but not statistically different to either 3 or 6 months ART.

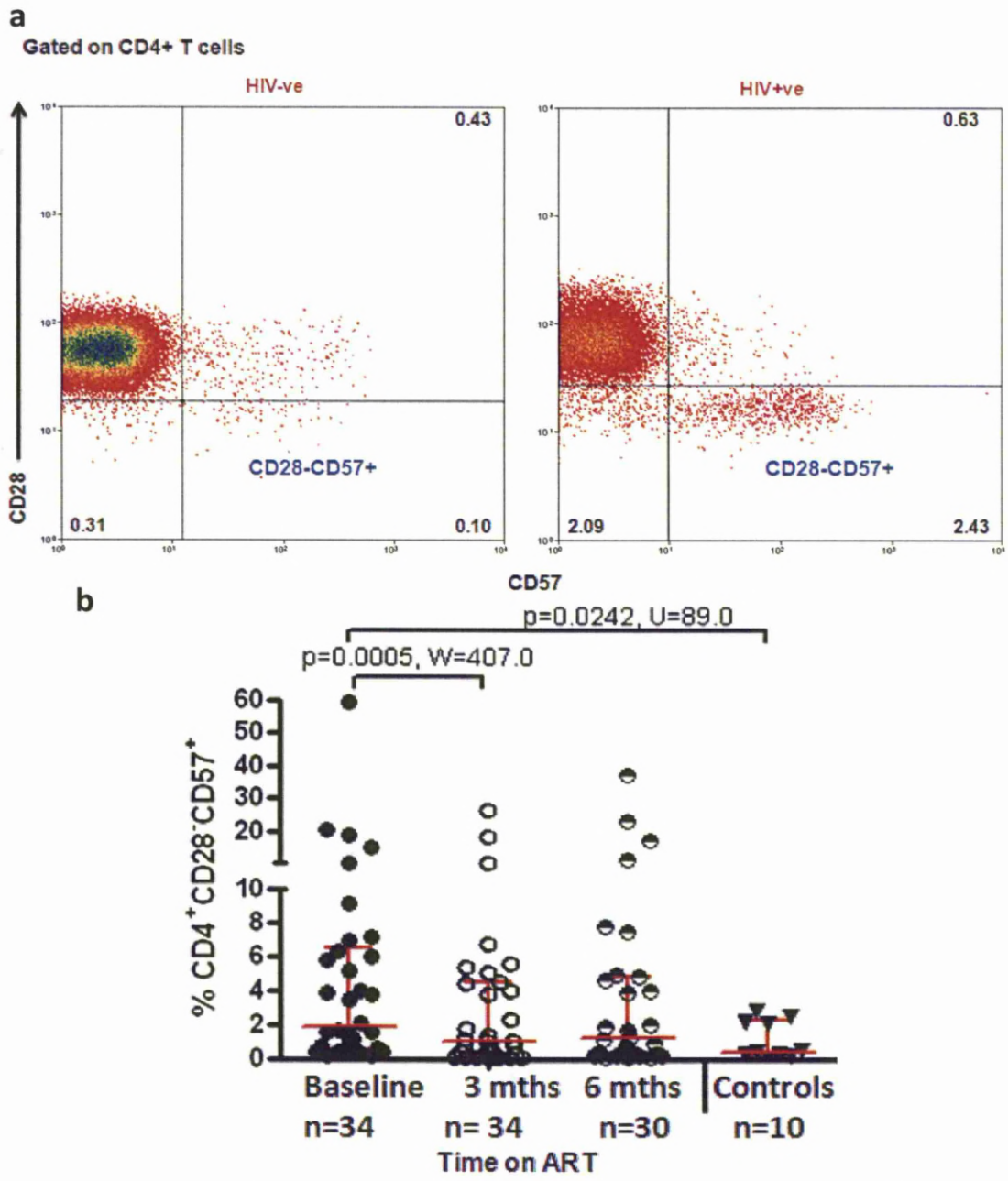


Figure 5.5|Phenotypic analysis of Senescent T cells in peripheral blood of adults on antiretroviral therapy a| Representative phenotypic analysis of CD28 and CD57 expression in peripheral blood CD4+ T cells **b|** The frequency of CD28-CD57+ senescent cells in PB of HIV negative and infected persons on ART. Red horizontal bars represent median values of senescent T cells. Statistical significance was analysed using the Wilcoxon matched pairs when comparing CD4+ T cell counts of HIV-infected persons on ART, and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison.

5.3.3 Functional analysis of CD4 T cells in peripheral blood of adults on antiretroviral therapy

Having established that the numbers of circulating effector memory and central memory (T_{CM}) T cells were recovering during antiretroviral therapy, we sought to establish whether the functions of these cells were recovering as well. The function of the effector T cells was assessed using the T cell ELISpot assay while the function of central memory T cells was determined by assessing their proliferative capacity using the CFSE proliferation assay. In addition, we also assess the ability of CD4 T cells to express the CD154 molecule and produce cytokines using an intracellular cytokine assay.

5.3.3a Regeneration of pneumococcal-specific T cell ELISpot responses during antiretroviral therapy

In chapter 4 we demonstrated that pneumococcal-specific effector function measured by T cell ELISpot was compromised especially in individuals with CD4 count <350 cells/μl. Following initiation of therapy, the median number of pneumococcal (D39WT)-specific IFN-γ SFC/10^6 PBMC increased significantly in the first three months of therapy (Figure 5.6a; p=0.0388, W=222.0). This effector function did not increase further at six months of ART. The ELISpot responses were significantly lower compared to controls (median for controls: 24 SFC) at 6 months ART (p=0.0244, U=57.00). Although, there was an increase in the median number of responding cells following stimulation with pneumolysin deficient mutant (D39Ply-)(Figure 5.6b; median: 3 months 3 SFC, 6 months 3.5 SFC and controls 41 SFC), the difference was not statistically significant even at 6 months ART (p=0.1304, W=-82.00). The ELISpot responses were still significantly lower compared with controls at 6 months ART (p=0.0006, U=26.00). There was no recovery in the ELISpot responses to the positive control antigens PPD or influenza vaccine (Figures 5.6c and d).

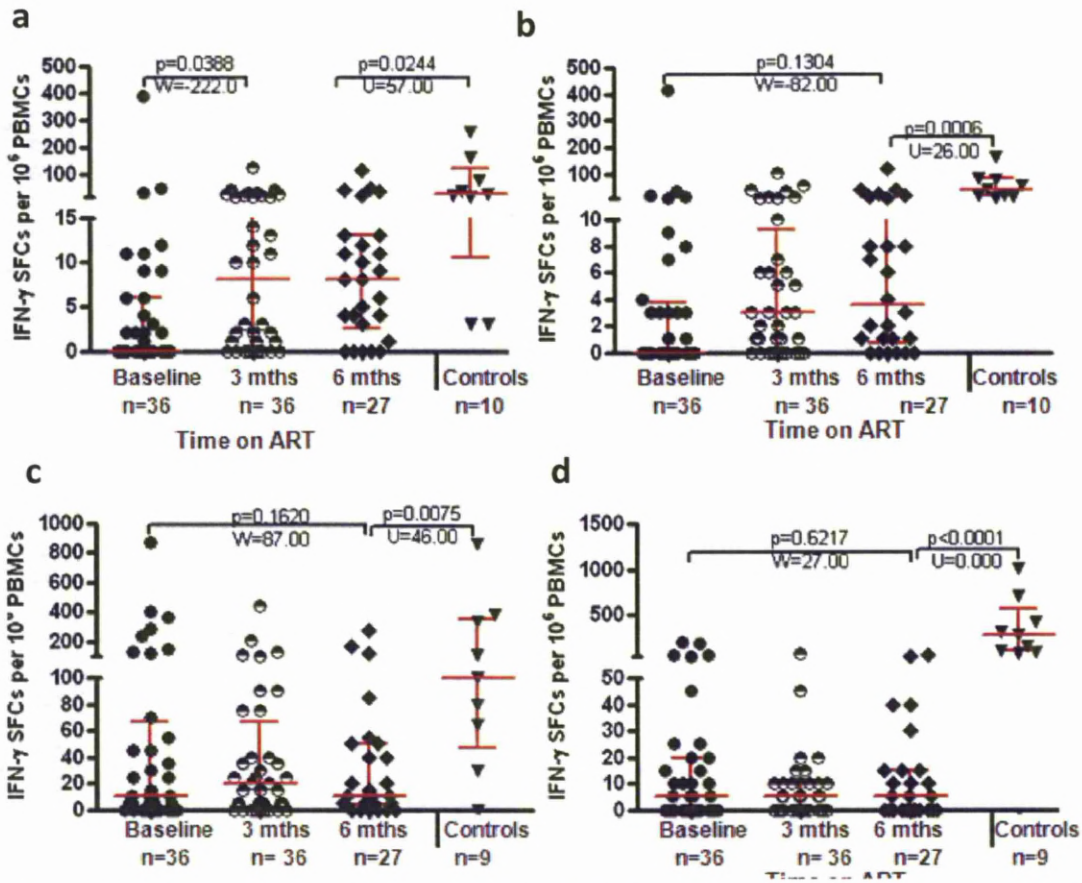


Figure 5.6 | *Ex vivo* interferon-gamma (IFN-γ) ELISpot responses to pneumococcal protein antigens after overnight stimulation of PBMCs of adults on antiretroviral therapy. IFN-γ ELispot response to **a|** pneumoCCS derived from a WT pneumococcal strain (D39) **b|** pneumoCCS derived from a Ply negative (-) D39 mutant **c and d|** positive control antigens *M. tuberculosis* PPD and influenza vaccine respectively. Black horizontal bars represent median values of total responses minus background. Statistical significance was analysed using the Wilcoxon matched pairs when comparing CD4+ T cell counts of HIV-infected persons on ART, and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison. Graph represents median values of CD4 T cell responses minus background.

5.3.3b CD4+ T cell proliferative responses to pneumococcal antigens during antiretroviral therapy

Following the initiation of ART, the median proportion of proliferating CD4+ T cells in response to the pneumoCCS derived from a WT pneumococcal strain (D39WT) increased from 2.197% (IQR, 0.1898-5.118) at baseline to 5.950% (3.109-7.652) at 6 month ART (Figure 5.7a, p=0.0384, W=-61.00). No significant differences between 6 months ART and HIV negative controls were observed (p=0.6606, U=62.00). We observed no significant differences between HIV-infected patients on ART and HIV-uninfected control subjects following stimulation with the pneumoCCS derived from a Ply negative (-) D39 mutant (Figure 5.7b). There was no significant increase in the proliferative capacity of the cells from HIV-infected patients in response to the positive control antigen influenza after 6 months of therapy (Figure 5.7c, median: 2.418% [IQR, 0-10.42] at baseline and 3.304% [0.5475-8.340] p=0.3399) at 6 months ART. The proliferative capacity of the CD4+ T cells (in response to Flu antigen) remained significantly lower than that of HIV-uninfected control subjects (p=0.0346, U=33.50).

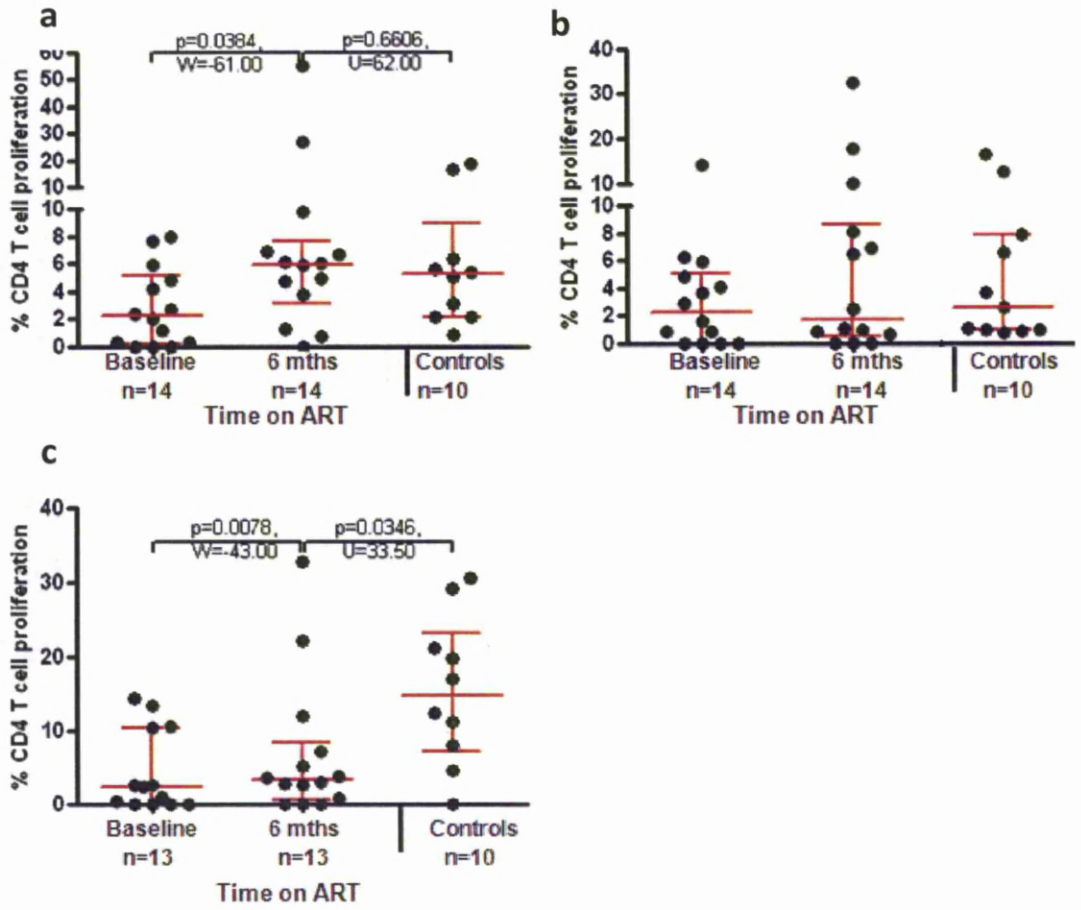


Figure 5.7 | Proliferative capacity of pneumococcal-specific CD4+ T cells during antiretroviral therapy. Percentage of proliferating CD4+ T cells in response to **a|** pneumoCCS derived from a WT pneumococcal strain (D39) **b|** pneumoCCS derived from a Ply negative (-) D39 mutant **c|** positive antigen control Influenza vaccine at baseline, month 3, 6 and controls. Red horizontal bars represent median values. Statistical significance was analysed using the Wilcoxon matched pairs when comparing CD4+ T cell counts of HIV-infected persons on ART, and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison. Graph represents median values of CD4 T cell proliferation minus background.

5.3.3c Restoration of the induction of CD154 (CD40L) on activated CD4 T cells in response to pneumococcal antigens during antiretroviral therapy

Having shown that the expression of CD154 on activated CD4+ T cells in response to pneumococcal protein antigens is impaired in asymptomatic HIV infected individuals (chapter 4 section 4.3.4), we sought to ascertain whether antiretroviral therapy can restore this function. Frequencies of activated CD4+ T cells expressing CD154 tended to be higher at 6 month ART compared baseline following stimulation with pneumoCCS derived from a WT pneumococcal strain (D39WT) (Figure 5.8a; p=0.05). No significant differences were observed between 6 month ART and the control group (p=0.57). The proportions of CD154 expressing cells was similar between HIV-infected persons on ART and controls in response to the pneumoCCS derived from a Ply negative (-) D39 mutant (Figure 5.8b; p=0.2838[6 months ART versus control group]). There was no recovery in CD154 expression with regard to the control antigens *M. tuberculosis* PPD (Figure 5.8c, p=0.3643, W=-46.00). However, there was recovery of Influenza responses after 6 months of ART (Figure d; p=0.0379, W=-160.0). CD154 expression in response to PPD and influenza after 6 months of ART remained significantly lower compared with controls (p=0.0114, U=80.50 and 0.0042, U=67.50 respectively).

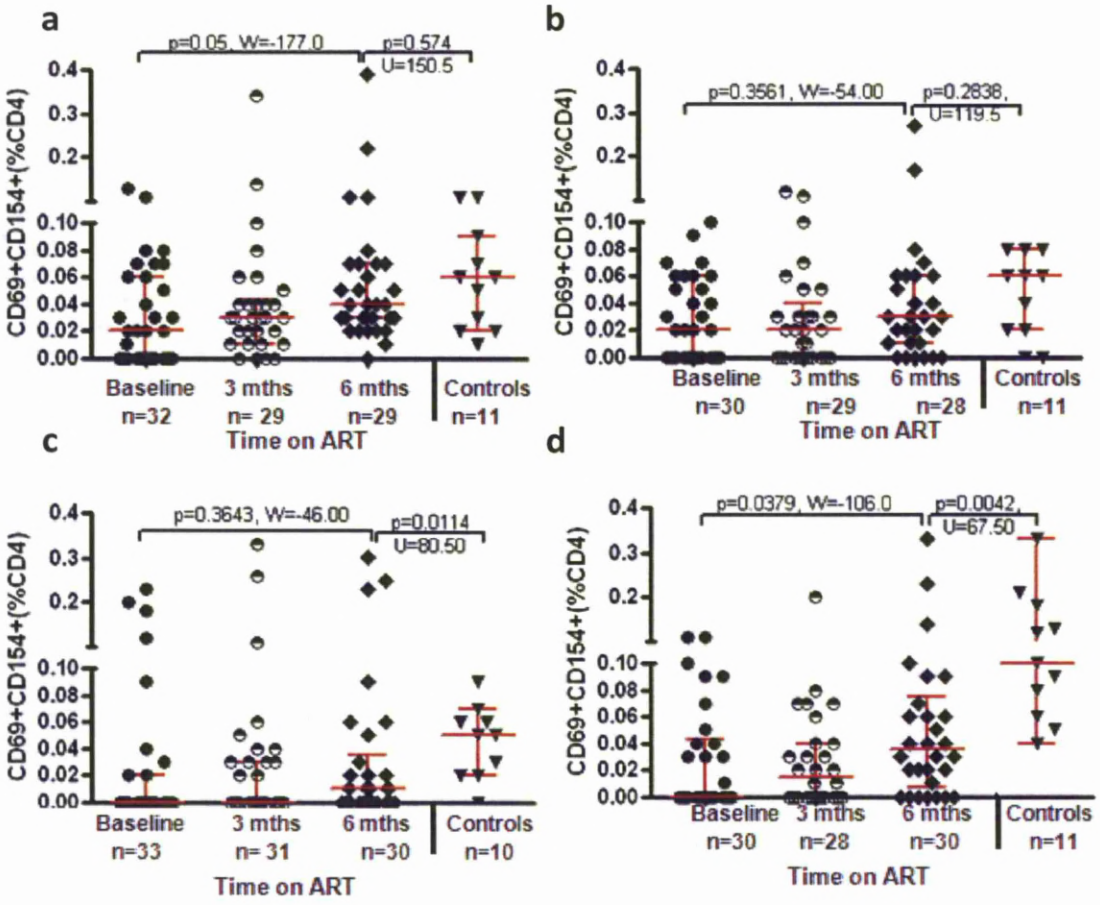


Figure 5.8 | Induction of CD154 (CD40L) on activated CD4 T cells from adults on ART. Induction of CD154 in response to **a|** pneumoCCS derived from a WT pneumococcal strain (D39WT) **b|** pneumoCCS derived from a Ply negative (-) D39 mutant **c an d|** positive antigen control *M. tuberculosis* PPD and influenza respectively. Red horizontal bars represent median values of total responses minus background. Statistical significance was analysed using the Wilcoxon matched pairs when comparing CD4+ T cell counts of HIV-infected persons on ART, and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison.

5.3.3d Cytokine-producing CD4+ T cells during antiretroviral therapy

Percentage cytokine secreting T cells (IFN-γ, IL-2 and TNF-α) in peripheral blood before and after 6 months ART compared to HIV-uninfected adults

The percentage of cytokine-producing $CD4^+$ T cells (IFN-γ, IL-2 and TNF-α) was used to assess the frequency of antigen-specific cells before and after 6 months ART. There was no difference in the total percentage of D39WT-specific peripheral blood $CD4^+$ T cells between pre-therapy and 6 months ART (median, 0.0990 vs. 0.0790%; p=0.4210, W=35.00; Figure 5.9a) and there was no difference between 6 months ART and controls (median, 0.0790 vs. 0.0740%; p=0.5136; Figure 5.9a). Similar results were observed for D39 pneumolysin mutant (median, 0.0610 vs. 0.1120 vs. 0.0550, p=0.4212, W=-40.00 [baseline vs. 6 months ART]; p=0.0644 [6 months ART vs. controls]) (Figure 5.9b). Frequencies of Flu-specific CD4 T cells were similar between baseline and 6 months ART (median, 0.0495 vs. 0.0610; p=0.3786, W=-32.00) but was lower compared to controls at 6 months ART (median, 0.0610 vs. 0.1700%; p=0.0432, U=38.50); Figure 5.9c).

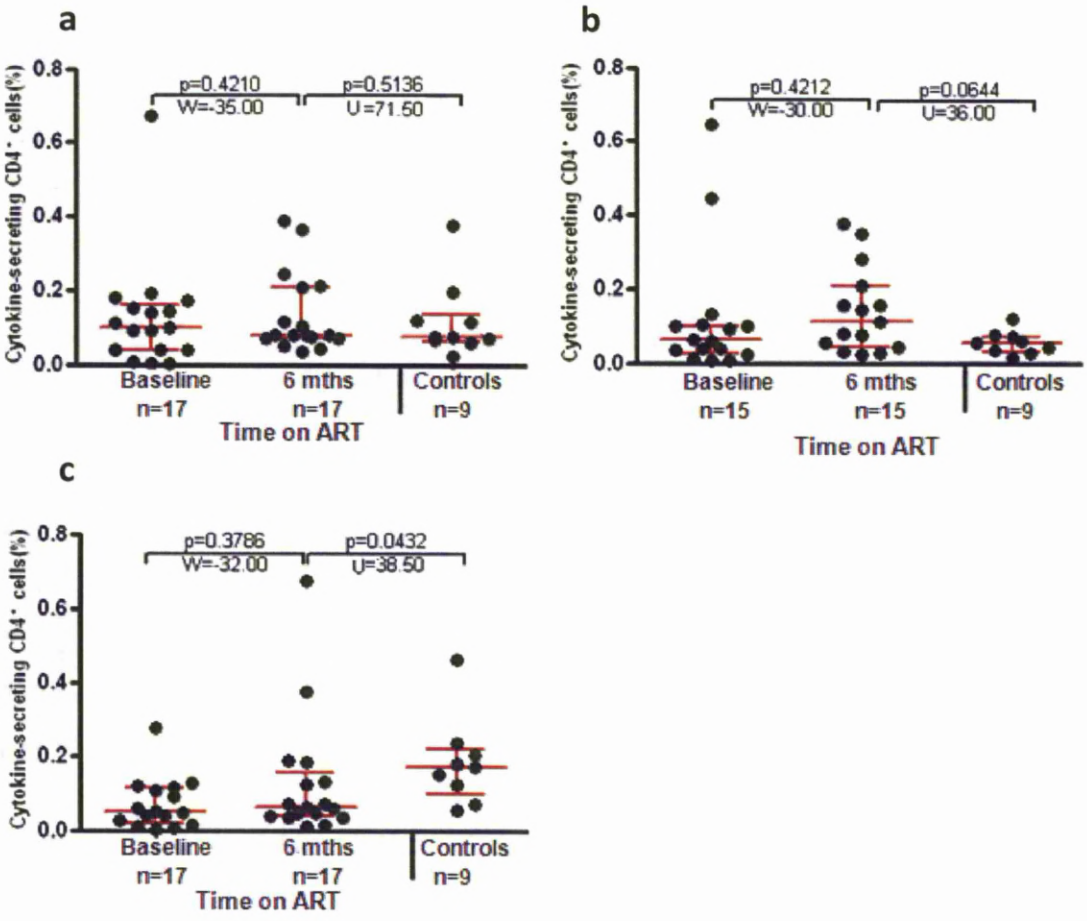


Figure 5.9 | No change in the frequency of antigen-specific CD4+ T cells before and after ART Percentage of cytokine-producing $CD4^+$ T cells (IFN-γ,TNF-α and IL-2) in response to **a|** pneumoCCS derived from a WT pneumococcal strain (D39WT) **b|** pneumoCCS derived from a Ply negative (-) D39 mutant **c|** positive antigen control influenza vacciine. Red horizontal bars represent median values of total responses minus background. Statistical significance was analysed using the Wilcoxon matched pairs when comparing CD4+ T cell counts of HIV-infected persons on ART, and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison. Analysis was done using data analysis programs PESTLE and SPICE (Simplified Presentation of Incredibly Complex Evaluations)

Analysis of multifunctional profile of CD4+ T cells before and at 6 months ART

Based upon the inability to simultaneously produce effector cytokines, dysfunction of T cells is a hallmark of the chronic phase of HIV infection (Betts, Nason et al. 2006). With the reduction of the viral load and increase in CD4+ T cell levels after initiation of antiretroviral therapy, it was hypothesized that there would be a change in the ability of CD4+ T cells to simultaneously produce effector cytokines. The relative proportions of pneumococcal-specific monofunctional (cells producing one cytokine), bifunctional (two cytokines), and polyfunctional CD4 T cells (three cytokines) were evaluated at baseline, 6 months ART and in HIV-uninfected controls. At baseline, D39WT- and D39Ply-specific responses were either mono or bifunctional with more than 75% of these cells monofunctional (Figure 5.10). At six months ART over 25% of the cells were bifunctional with about 2% of cells coexpressing three cytokines. The cells from HIV-negative persons coexpressed two or three cytokines. For the control antigen Flu, nearly all cells expressed a single cytokine at baseline. At six months ART about 25% of the cells were producing two cytokines (Figure 5.10).

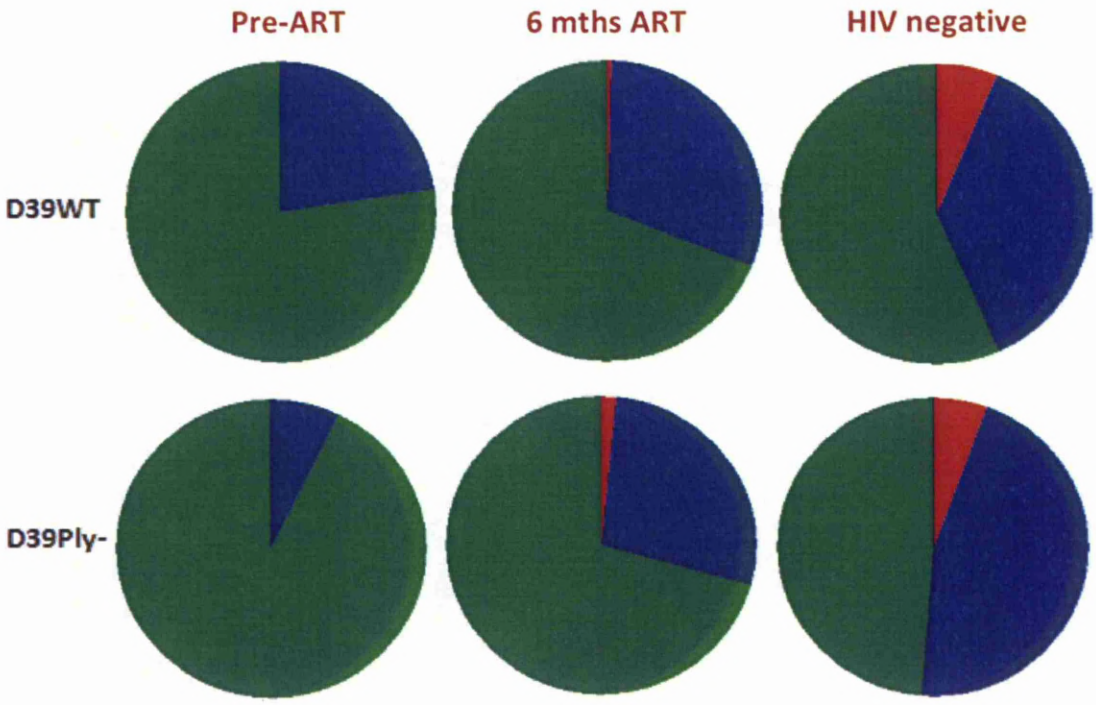


Figure 5.10 | Polyfunctional CD4+ T cell profile pre-ART, 6 months ART and HIV-uninfected controls. Cytokine positive CD4+ T cells were analyzed for different combinations of IFN-γ TNF-α, and IL-2. Pie charts represents the median proportions of D39WT, D39Ply- and Flu-specific polyfunctional (cells producing three cytokines, ***red***), bifunctional (cells producing two cytokines, ***blue***) and monofunctional (cells producing one cytokine, ***green***) out of the total cytokine CD4 T-cell response. Graphical representations of T-cell responses (using background-deducted flow cytometric data) was generated using data analysis programs PESTLE and SPICE (Simplified Presentation of Incredibly Complex Evaluations).

5.3.4 Phenotypic Analysis of B Cells

5.3.4a Memory B cells

Memory B cells are central to the swift amplified immune response observed following re-exposure to an antigen (Crotty, Aubert et al. 2004; Pollard, Perrett et al. 2009). This is demonstrated by a significant increase in antigen-specific B cells in the bone marrow and spleen following immunisation or exposure to a pathogen. They are involved in the maintenance of the supply of long-lived plasma cells to maintain long-term antibody levels even when there is no re-exposure to antigen (Slifka, Antia et al. 1998; Bernasconi, Traggiai et al. 2002). Memory B cells are generated in histological structures called the germinal centres (Vinuesa, Sze et al. 2003). In the germinal centres, naïve B cells mature into antibody-secreting plasma cells or memory B cells after activation, proliferation, somatic hypermutation of rearranged V region genes, isotype switching of immunoglobulin, and antigen selection (Rajewsky 1998; Vinuesa, Sze et al. 2003).

Memory B cells co-express cell surface receptors; CD19 and CD27. Additionally, they express somatically mutated IgG, IgA, IgM or IgD receptors (Maurer, Fischer et al. 1992; Klein, Rajewsky et al. 1998; Tangye, Liu et al. 1998; Agematsu, Hokibara et al. 2000). Memory B cells can be divided into two subsets, namely isotype-switched and IgM memory B cells. Isotype-switched memory B cells ($CD19^+$, $CD27^+$ IgM^- IgD^-), express IgG or IgA receptors and differentiate into plasma cells secreting IgG, IgE or IgA. The other subset, IgM memory B cells ($CD19^+$, $CD27^+$, IgM^+ IgD^+) express IgM somatically mutated variable region genes.

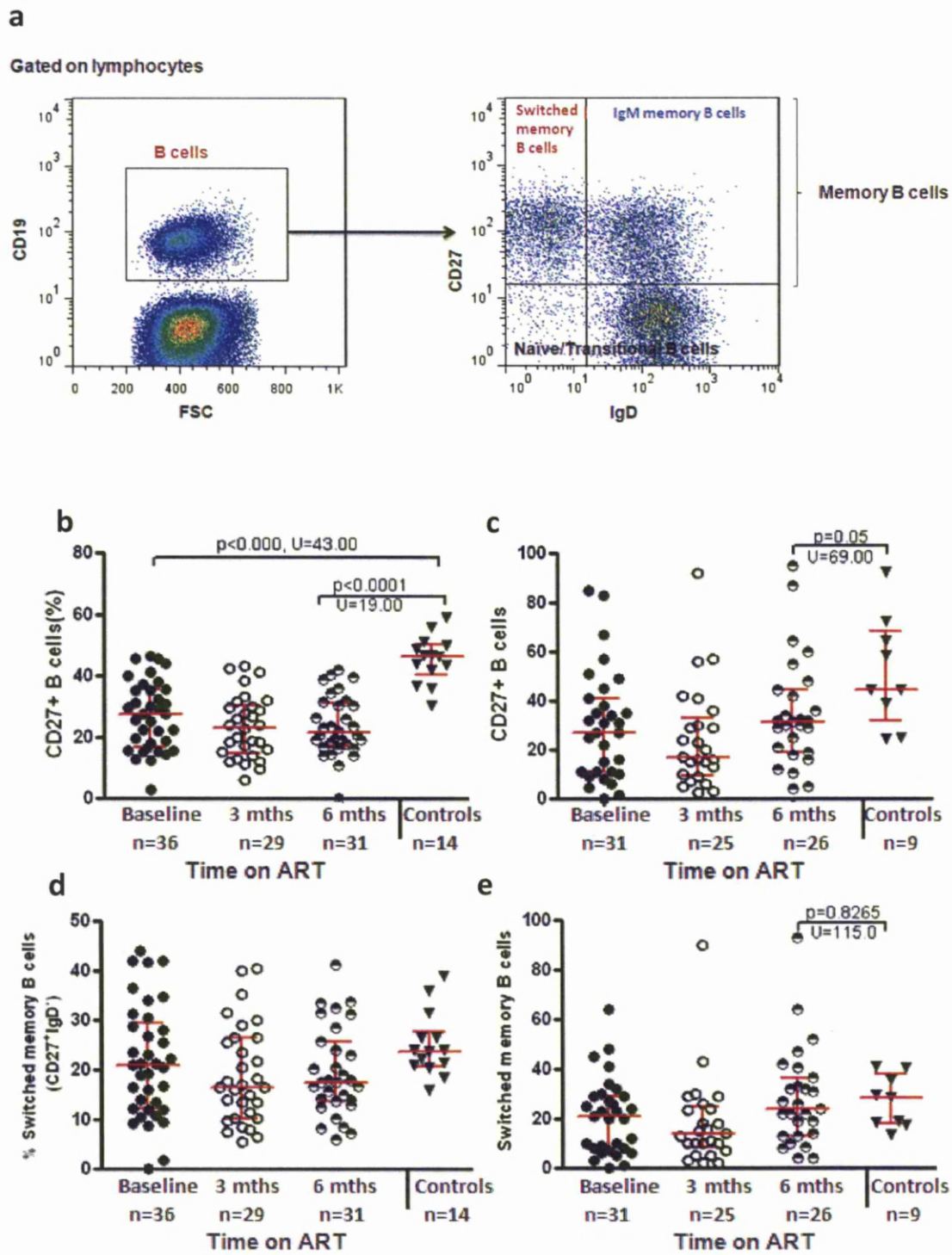
IgM memory B cells have a prediversified IgM antigen receptor and are capable of responding immediately to the antigens of encapsulated bacteria (such as *S. pneumoniae*) in a T-cell-independent fashion (Kruetzmann, Rosado et al. 2003; Weller, Braun et al. 2004; Shi, Yamazaki et al. 2005). On the other hand, switched memory B cells require T-cell co-stimulation to produce IgG and other isotypes of antibody and are known to promote long-term serological memory. (Klein, Rajewsky et al. 1998; Shi, Agematsu et al. 2003; Hart, Steel et al. 2007). Peripheral IgM memory B cells appear to be the circulating correlate of marginal zone B cells in the spleen (Moir and Fauci 2009).

IgM memory B cells are thought to be important in immunity against pneumococcal infection as they have been shown to produce high-affinity IgM antibodies early in the course of pneumococcal infection which may restrict the replication of the bacteria in circulation (Shi, Yamazaki et al. 2005; Hart, Steel et al. 2007). Enumerating the numbers of antigen-specific memory B cells may serve as a surrogate of immune memory, since immune responses following vaccination are evident even when antigen-specific antibodies are no longer evident (Nanan, Heinrich et al. 2001).

5.3.4b Memory B cells in adults during ART

As stated in the introduction of this chapter, HIV infection is associated with a wide range of B cell defects including reduction of CD19+CD27+ memory B cells. To evaluate the effects of ART on memory B cells, absolute counts and percentages of memory B cells defined as CD19+CD27+ lymphocytes were determined at baseline, month 3 and 6 of therapy and in control subjects at month 6 of the study. Additionally, IgM memory B cells (CD27+IgD+) and switched memory B cells (CD27+IgD-) as percentages of CD19+CD27+ cells were evaluated. During the 6 months of antiretroviral therapy, the percentages of memory B cells (CD19+CD27+) in the patients remained relatively unchanged and significantly lower than in the controls (Figure 5.11b; baseline median(range) 27.29% [2.82–46·42], 3 months 23·09% [5·95 – 43·19] and 6 months 20.65% [13.71– 41·80%], p<0.0001, U=19.00]. The absolute numbers of memory B cells remained relatively unchanged during the six months of ART and tended to be lower compared with HIV negative subjects (Figure 5.11c p=0.05).

A similar analysis was performed for isotype switched and IgM memory B cells (Figures 5.11d,e,f and g). Consistent with the data generated in the laboratory by Dr Oluwadamilola H Unuigbe-Iwajomo (PhD thesis 2011), we found that the relative size and the absolute numbers of the isotype switched memory B cells remain unchanged during HIV infection (Figure5.11d and e). The proportion and absolute numbers of the IgM memory B cells in the patients remained relatively unchanged during the 6 months of ART and remained significantly lower compared to HIV negative subjects (Figures 5.11f and g; p<0.0001, U=18.00 and p=0.0003, U=22.00].


a
Gated on lymphocytes
B cells
CD19
FSC
Switched memory B cells
IgM memory B cells
Memory B cells
CD27
IgD
Naive/Transitional B cells
b
p<0.000, U=43.00
p<0.0001 U=19.00
CD27+ B cells(%)
Baseline n=36
3 mths n=29
6 mths n=31
Controls n=14
Time on ART
c
p=0.05 U=69.00
CD27+ B cells
Baseline n=31
3 mths n=25
6 mths n=26
Controls n=9
Time on ART
d
% Switched memory B cells (CD27+IgD-)
Baseline n=36
3 mths n=29
6 mths n=31
Controls n=14
Time on ART
e
p=0.8265 U=115.0
Switched memory B cells
Baseline n=31
3 mths n=25
6 mths n=26
Controls n=9
Time on ART

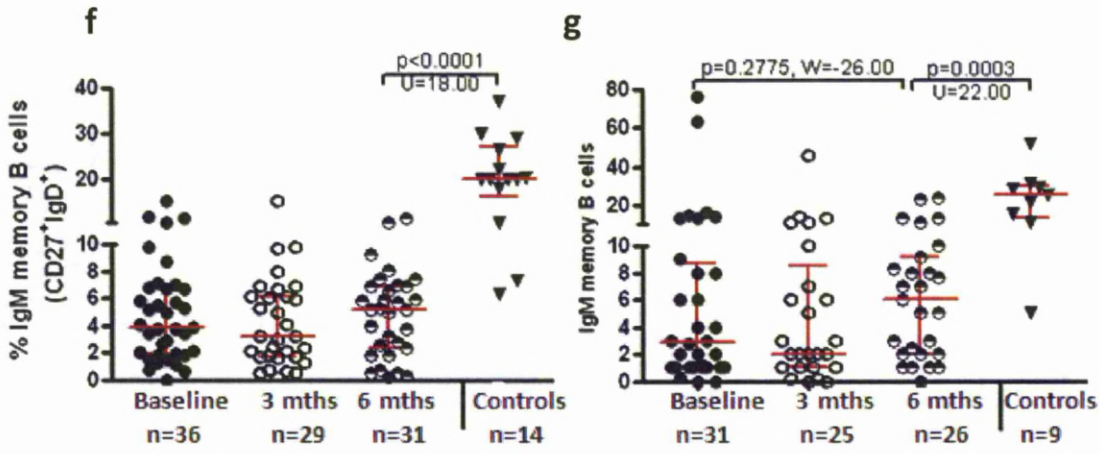


Figure 5.11|Phenotypic analysis of memory B cells in peripheral blood of adults on antiretroviral therapy a| Representative phenotypic analysis of memory B cells in peripheral blood B (CD19+) cells, **b and c|** The frequency and absolute numbers of total memory B cells in PB of HIV negative and infected persons on ART, **d and e|** The frequency and absolute numbers of isotype-switched memory B cells in PB of HIV negative and infected persons on ART, **f and g|** The frequency and absolute numbers of IgM memory B cells in PB of HIV negative and infected persons on ART. Red horizontal bars represent median values of B subsets. Statistical significance was analysed using the Wilcoxon matched pairs when comparing CD4+ T cell counts of HIV-infected persons on ART, and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison

5.3.5 Functional analysis of B cells in peripheral blood of adults on antiretroviral therapy

5.3.5a Pneumococcal specific B cell responses in HIV-1-infected Adults on ART

Work in this laboratory established that memory B cell responses to some pneumococcal protein antigens (e.g. CbpA) are compromised in HIV-infected individuals (JID 2011, *In Press*). We therefore sought to determine whether memory B cell responses to pneumococcal protein antigens recover upon starting antiretroviral treatment. The pneumococcal protein - specific antibody-secreting cells were enumerated by B cell enzyme-linked immunosorbent spot (ELISpot) as described in Chapter 2 materials and methods (section 2.8). The cells were stimulated with pneumococcal proteins: CbpA, PspA, PsaA and PdB. The median numbers of IgG producing memory B cells to CbpA, PspA and PsaA remain relatively changed following initiation of antiretroviral therapy. At 6 months ART, the median number of IgG producing memory B cells to CbpA (1 SFC/10^6 PBMC[IQR, 0-6]) was still significantly lower compared with controls (5 SFC/10^6 PBMC[IQR, 1-19], p=0.0408, U=68.00)(Figure 5.12a). No significant differences between memory B cell responses to PspA in HIV-infected patients at 6 months ART and HIV negative subjects (Figure 5.11b, p= 0.6599, U=89.00). The memory B cell responses to PsaA and PdB were significantly lower at 6 months ART compared to healthy controls (Figures 5.12c and d, p=0.0224, U=56.50 and p=0.0366, U=51.50 respectively). It should, however, be emphasised that the PsaA data must be treated with caution because of the low numbers of responding memory B cells.

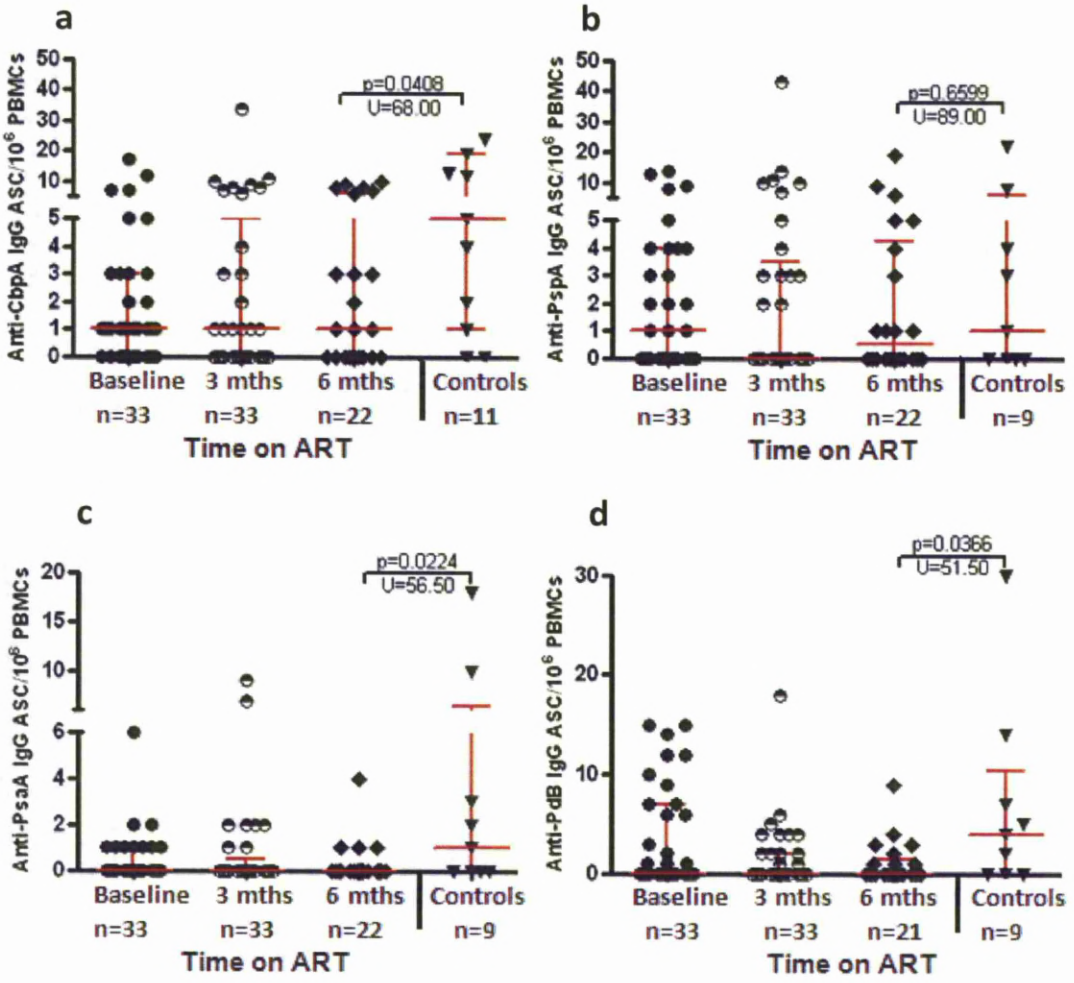


Figure 5.12 | Memory B cell responses to pneumococcal protein antigens during antiretroviral therapy. Memory B cell response to **a|** CbpA **b|** PspA **c| PsaA d|PdB**. Red horizontal bars represent median values of total responses minus background. Statistical significance was analysed using the Wilcoxon matched pairs when comparing CD4+ T cell counts of HIV-infected persons on ART, and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison.

5.4 Discussion

Initiation of antiretroviral therapy leads to a reduction in HIV-1 related opportunistic infections and a reduction in the incidence and magnitude of IPD (Kaplan, Hanson et al. 2000; Grau, Pallares et al. 2005). Whether pneumococcal- specific protective immunity returns and how naturally-acquired pneumococcal immunity changes with ART immune reconstitution is unknown. To examine the dynamics of immune reconstitution (in the context of pneumococcal immunity), we carried out a detailed assessment of CD4 and memory B cells subsets in peripheral blood of HIV-infected adults initiated on antiretroviral therapy and examined their functional capacity when stimulated with pneumococcal protein antigens and compared them with uninfected adults. We show that in addition to the recovery of CD4+ T cells and to a limited extent, memory B cells, there is some restoration of pneumococcal-specific CD4+ T cell responses by 6 months ART but not pneumococcal-specific B cell memory.

Contrary to our expectations, pneumococcal carriage rates did not decrease during the 6 months of ART but were not significantly increased at both 3 and 6 months ART, although no. These results are unlikely to be due to how the samples were collected or processed. The nasopharyngeal swabs were taken by the same and well experienced individuals over the six months study period. The apparent increase in the carriage rates may be due to seasonal variation. Baseline samples were taken in December and January (summer months) while the six month follow-up samples were taken in June and July (winter months). There is an increased chance of person-to-person spread of the pneumococcus during winter because of colds and influenza. However, the pneumococcal carriage rate of the HIV negative subjects at 6 month (taken in winter months) of the study was 10% which was comparable to the rate observed in chapter 4 where the samples were taken (mainly) during the summer months. The serotypes of the *S. pneumoniae* isolates have not been identified yet and as such we have not been able to determine whether ART affect the serotype(s) of the colonising *S. pneumoniae*.

Following the initiation of antiretroviral therapy and as expected we observed a rapid increase in the CD4 T cell counts in the first three months of therapy. This is consistent with previous observations that showed that during the first 8-12 weeks of therapy, there is a steep increase in circulating CD4+ T cells. The studies further showed that reconstitution of circulating CD4+ T cells occurs in two phases: a rapid increase in the first 8 to12 weeks followed by a more gradual increase in CD4 T cell counts (Lederman 2001; Gandhi, Spritzler et al. 2006; Robbins, Spritzler et al. 2009). The rapid first phase of CD4+ T cell recovery coincides with the rapid reduction in activated CD4+ cell populations (Robbins, Spritzler et al. 2009). The increase in the CD4+T cell counts was observed in nearly all patients regardless of their baseline CD4 T cell counts. Several other studies have shown that the net yearly increases in CD4 T cell counts are independent of baseline CD4 T cell counts (Hunt, Deeks et al. 2003) and ART regimen assignment (Robbins, Spritzler et al. 2009).

As stated in the introduction of this chapter, data from a number of studies suggest that patients who start therapy with low nadir CD4 T cells are less likely to adequately repopulate CD4 T cells even if they achieve long-term viral suppression. In this study, 68% of the patients started therapy with CD4 T cell count less than 200 cells/ul and the data suggest that most of them may have achieved viral suppression by six months ART. It remains to be seen therefore whether these individuals will adequately repopulate CD4 T cells after long-term viral suppression and also experience fewer episodes of opportunistic infections (French, Keane et al. 2007). Only one study has reported normalization of total CD4 T cell numbers in adults who started therapy with low baseline CD4 T cell counts (<200 cells/μl) and this was after at least 7 years of therapy (Vrisekoop, van Gent et al. 2008).

The relative size of naive, central and effector memory CD4+ T cells remained unchanged after 6 months of therapy. There were however modest increases in the absolute counts of naive, central and effector memory cells over the 6 months of therapy. Studies done in the last decade have demonstrated reconstitution of CD4+ T cell subsets within weeks of initiation of therapy. Using data from 978 patients (621 with comprehensive immunological assessments) from the AIDS [Acquired Immunodeficiency Syndrome] Clinical Trials Group protocol 384, a randomized international trial of initial ART, Robbins *et al.* showed that ART reconstitutes T cell subsets (naive and memory T cells) regardless of baseline CD4+ T cell count (Robbins, Spritzler et al. 2009). However, the degree of reconstitution of was dependent on baseline CD4+ cell counts.

Although, we did not determine which subset of CD4 T cells reconstitute first, studies have shown that the reconstitution occurs in two phases. During first phase of CD4 T cell reconstitution (~8-12 weeks), the increase in CD4+ T cells is primarily an increase in the cells of the memory phenotype (Lederman 2001; Moore and Keruly 2007; Robbins, Spritzler et al. 2009) and the naive CD4 reconstitute mainly in the second phase of the cellular increase. Thus CD4 T cells of the memory phenotype generally reconstitute earlier than naive T cells. Additionally, in chapter 4 of this thesis we demonstrated that effector memory T cells decline at a slower rate compared to central memory CD4+ T cells during HIV infection, it is not clear from the data obtained in this study as to which subset of memory T cells reconstitutes first during ART.

As stated earlier, patients with low baseline CD4 T cell count (some studies used CD4$\leq$ 200 cells/μl as a definition of low baseline CD4 T cell count while others used $\leq$ 350 cells/μl) at the start of therapy tend to have smaller increases in naive T cells and greater increases in memory T cells resulting in persistently abnormal absolute cell counts and CD4+ naive-memory cell ratios (Vrisekoop, van Gent et al. 2008; Robbins, Spritzler et al. 2009). With regard to our study, it is too soon to assess whether there are greater increases in the memory T cells compared to naive T cells. The study participants are probably in a transition phase: from the first phase (which is characterised mainly by reconstitution of memory CD4+ T cells) moving into the second phase of reconstitution (which is characterised mainly by reconstitution of naive CD4+ T cells).

In chapter 4 we showed that Tregs expand as disease progresses. Data here show that the Tregs (measured as CD4+CD25^{hi}FoxP3+) contracted during the first 6 months of ART. This may be due to a decrease in immune activation (Robbins, Spritzler et al. 2009) which in advanced disease probably causes the expansion of the Treg population as an attempt to slow down disease progression. Others however have shown (using different phenotypic identification methods of Tregs such as CD25CD127^{low}CD4 and CD4CD25^{hi}) that ART has little influence on the proportion of Tregs (Lim, Tan et al. 2007; Gaardbo, Nielsen et al. 2008).

In addition to CD4 T cell recovery and reduction in the relative size of Tregs, there was a reduction in the senescence levels of CD4 T cells. Chronic antigenic stimulation and immune activation during HIV infection are thought to accelerate T cell differentiation resulting in cells that eventually lose expression of CD28 and increase the expression of CD57 (Appay and Sauce 2008; Cao, Jamieson et al. 2009; Desai and Landay 2010). Since antiretroviral therapy reduces the antigen load (by extension antigenic stimulation) and immune activation this may partly explain reduction in the senescence levels of CD4+ T cells.

Our study indicates that by 6 months ART there are signs of regeneration of pneumococcal specific CD4+ T cell responses including effector memory responses (*ex vivo* IFN-γ ELISpot responses), ability to proliferate (central memory responses), ability to express the costimulatory molecule CD154 and ability to produce simultaneously multiple cytokines (polyfunctional capacity). Several studies have demonstrated that antigen specific CD4+ T cell responses to prevalent antigens (e.g. candida, cytomegalovirus and mycobacterial antigens) are readily restored following initiation of therapy and suppression of viral replication (Lange, Valdez et al. 2002; Keane, Price et al. 2004; Furco, Carmagnat et al. 2008; Sutherland, Young et al. 2010). Because of the high rates of nasopharyngeal carriage in Malawi, pneumococcal antigens can be considered prevalent antigens. Therefore, it is not surprising that by 6 months ART there is some restoration of immune responses to *S. pneumoniae*. It would be interesting however, to determine whether this restoration is sustained in the long-term.

Studies have also shown that for some antigens (e.g. CMV) restoration of CD4 T cell responses may be short-lived and least effective in severely immunodeficient patients (Lange, Valdez et al. 2002; Sieg, Mitchem et al. 2002; Lederman, Williams et al. 2003; Keane, Price et al. 2004; French, Keane et al. 2007). The data to be obtained at 12 months ART may shed some light on the robustness and durability of the regenerated immunity against pneumcoccal T-cell dependent antigens.

HIV infection leads to loss of memory B cells. In our study, the relative size and the absolute numbers of memory B cells remained unchanged after six months of therapy and remained lower compared to controls. Studies elsewhere have also shown that the increase in the percentage and number of CD27-expressing resting B cells (classic resting memory B cells) following initiation of therapy occurs slowly and fail to normalise (De Milito, Morch et al. 2001; Chong, Ikematsu et al. 2004; D'Orsogna, Krueger et al. 2007). Consistent with the work done in this laboratory by Dr Oluwadamilola H. Iwajomo, the relative size of the population of switched-memory B cells was not affected by HIV infection but there was loss of IgM memory B cells in HIV-infected individuals (Iwajomo, In press). At 6 months ART the proportion and absolute numbers of the marginal memory B cells remained significantly lower compared with HIV negative subjects. Marginal zone B cells are thought to play a protective role against infection with encapsulated bacteria including *S. pneumoniae* (Martin, Oliver et al. 2001) (Martin and Kearney 2002; Kruetzmann, Rosado et al. 2003). Failure of the marginal zone memory B cells to expand may partly explain why HIV-infected persons on treatment are still at a higher risk of pneumococcal infections compared to HIV-uninfected individuals (Heffernan, Barrett et al. 2005).

In contrast to CD4+ T cell responses, there was no regeneration of memory B cell responses to T-cell dependent pneumococcal antigens at 6 months ART. Recent studies on reconstitution of immunity to some T-cell-dependent antigens (for example, influenza virus, measles and tetanus toxoid) indicated that restoration of memory B cell responses during antiretroviral therapy is either absent or incomplete (Malaspina, Moir et al. 2005; Titanji, De Milito et al. 2006; Hart, Steel et al. 2007).

Failure to detect restoration (poor restoration) of B cell responses in our study may be due to the fact that at six months of therapy it is still early to observe any regeneration of antigen-specific memory B cell pools since at this stage memory B cells have not started recovering. In some cases, the number of antigen-specific memory B cells may be very low to detect any restoration. Furthermore, lack of restoration of antigen-specific memory B cells may be due to defects in the CD4+ T cell compartment which have not been fully restored at 6 months ART. For instance, CD4+ T-cell help given to B cells has been shown to be defective in HIV-viremic individuals as a result of an impaired interaction between the CD154(CD40L) on the T cells and CD40 on the B cells (Moir, Ogwaro et al. 2003). Patients in this study however will be follow-up for another six months to assess whether there is any regeneration of B cell responses to pneumococcal T cell dependent antigens at 12 months ART.

CHAPTER 6 IMMUNE RESPONSES TO PROTEIN ANTIGENS FOLLOWING PCV7 VACCINATION AMONG HIV-UNINFECTED AND INFECTED MALAWIAN ADULTS ON ART

6.1 Introduction

Evidence presented in previous chapters showed that HIV infection causes a range of immune defects which do not entirely resolve with ART. This may be reason why HIV-infected individuals on ART are still at an increased risk of invasive pneumococcal disease (IPD) compared to HIV-uninfected individuals. A recently completed study however showed that PCV7 vaccination prevented recurrent invasive pneumococcal disease in a cohort of HIV-infected Malawian adults (French, Gordon et al. 2010) suggesting that vaccines may offer a further option for protection against IPD in HIV-infected individuals. Having demonstrated (in previous chapters) that natural immunity to the pneumococcus is impaired we wondered whether this will be true of vaccine antigens. A study [capitalising on a recently completed vaccine trial in Malawian adults (French, Gordon et al. 2010)] was therefore designed to assess the effect of HIV infection on the immune memory mounted in response to vaccine antigens.

6.1.1 Rationale and Aims

In the light of the findings in chapter 4 and 5, the aim of this study was to define the robustness and durability of immune memory conferred by a vaccine antigen in the context of HIV infection and antiretroviral therapy. In the absence of pneumococcal protein vaccines, diphtheria toxoid (DT) [the carrier protein in the 7-valent pneumococcal conjugate vaccine [PCV7]) was used as a model vaccine antigen. Additionally, the study sought to determine whether there are any surrogate markers of long term memory conferred by vaccine antigens. In a population where carriage rates are high, naturally-induced pneumococcal T cell responses were used as a positive control.

The use of pneumococcal T cell responses as a positive control in this study offered an opportunity to: i) compare naturally-induced and vaccine induced surrogate markers, ii) assess the effects of vaccination on nasopharyngeal carriage rates and iii) study individuals well established on ART. *M. tuberculosis* PPD was also used as a another positive. PPD represents a model of T cell responses induced by vaccination with bacillus Calmette-Guerin live vaccine or presumably by sub-clinical infection with environmental mycobacteria-expressing antigens that cross-react with PPD (Oftung, Borka et al. 1998; Mustafa, Oftung et al. 2000). For B cell memory responses tetanus toxoid was used as a positive control.

6.1.2 Study design

Study participants were drawn from the placebo arm of a recently completed double-blind, randomised, placebo-controlled 7-valent pneumococcal conjugate vaccine trial in HIV-infected and uninfected Malawian adults (French, Gordon et al. 2010). All study participants had recovered from a previous episode of invasive pneumococcal disease, either bacteraemia or meningitis (a criterion for enrolment into the original vaccine trial). Additionally, all HIV-infected individuals were on antiretroviral therapy (as the national roll-out of ART commenced during the course of the vaccine trial). 15 HIV-uninfected and 32 HIV-infected previous placebo recipients were recruited into the study. Two doses of PCV7 were given intramuscularly in the left deltoid one month apart. The vaccinees were followed up for 5 months after the administration of the second dose of the vaccine.

As described in chapter 2 materials and methods (section 2.2.2) samples were collected at the time of enrolment (prior to administration of the vaccine), 1 month after the second dose and 5 months after the completion of a two dose vaccination schedule. The study was approved by the College of Medicine (University of Malawi) and Liverpool School of Tropical Medicine (LSTM) research ethics committees (Protocols: P.05/08.665 and 08.64 respectively).

6.2 Materials and Methods

6.2.1 General materials and methods are as described in chapter 2.

Antigen-specific T-cell immune responses were assessed using intracellular cytokine staining and expression of CD154 (chapter 2 materials and methods [sections 2.9 and 2.11]). Memory B cell responses to protein antigens were assessed using IgG B cell ELISpot (section 2.8). Peripheral blood T and B cell immunophenotypes were evaluated as described in chapter 2 materials and methods (section 2.5). Additionally, nasopharyngeal swabs were cultured to determine carriage rates. In this study we used whole pneumococcal protein antigens prepared from culture supernatants of a standard encapsulated type 2 strain (D39), diphtheria toxoid, positive antigen control *M. tuberculosis* PPD and tetanus toxoid as described in Chapter 2 materials and methods (section 2.4).

Whilst PCV7 is conjugated to diphtheria-derived carrier protein CRM_{197}, all measurements in this chapter were made to purified diphtheria toxoid which differs from CRM_{197} by one amino acid substitution. This work was done in collaboration with Dr Oluwadamilola H Unuigbe-Iwajomo (PhD Thesis 2011), who performed the B cell part of this study.

6.3 Results

6.3.1 Baseline characteristics of new PCV7 recipients

A total of forty-seven adults were recruited into the study: 15 HIV-uninfected median age 40 years (range 32-50), 13% of them were females and 32 HIV-infected individuals median age 40(range 34-47), 66% of them females (Table 6.1). All were clinically well at the time of the study without clinical evidence of ART failure. The median CD4 count for HIV-infected individuals was 382 (range 187-1043 cells/μl) which was significantly lower compared with HIV negative group (HIV negative: 719 [range 472-1410] p=0.0002). *S. pneumoniae* carriage was not detected in HIV-uninfected but was detected in 28% of HIV-infected participants (p=0.04 Fisher's exact test). As previously observed (in chapter 5), it appears that ART has no effect on pneumococcal carriage rates. All previously HIV negative study participants were tested for HIV to ensure they remained HIV seronegative.

Table 6.1| Baseline characteristics of new PCV7 recipients

	HIV-uninfected	HIV-infected	P*value
N	15	32	n/a
Female (%)	2/15(13)	21/32(66)	0.001
CD4, median (range)cells/μl	719(472-1410)	382(187-1043)	0.0002
Pneumococcal carriage (%)	0/15(0)	9/32(28)	0.04

*Statistical significance of differences between the HIV^- and HIV^+ adults was tested using Fisher's exact test (for sex and carriage) or Mann Whitney U test (for all others).

6.3.2 Pneumococcal nasopharyngeal rates in the study population during the study period

Nasopharyngeal carriage rates of *S. pneumoniae* in HIV-infected study participants remained relatively unchanged over the study period (Figure 6.1): baseline 28% (9/32), 2 months post vaccination 31% (9/29) and 6 months post vaccination 33% (9/27) suggesting that at this early time point, vaccination may not have any effect in the overall nasopharyngeal carriage rates in HIV-infected persons. However, it is not known whether the serotype of the colonising pneumococcus changed following administration of the vaccine as has been shown previously. Studies from the USA and the Gambia have shown that the introduction of PCV7 led to significant decrease in the carriage of vaccine serotypes (Whitney, Farley et al. 2003; Flannery, Schrag et al. 2004; Talbot, Poehling et al. 2004; Lexau, Lynfield et al. 2005; Millar, Watt et al. 2008) and an increase in disease incidence by non-vaccine serotypes (Eskola, Kilpi et al. 2001; Pai, Moore et al. 2005). Therefore, the high carriage rates in the HIV-infected group may be a result of increase carriage of non-vaccine serotypes following a decrease in vaccine serotypes. Unlike in the HIV-infected individuals, there was a transient increase in the carriage rate in HIV-negative persons: baseline 0% (0/15), 2 months post vaccination 23% (3/13) and 6 months post vaccination 7% (1/14). As before, without serotype information it is difficult to fully interpret this apparent change.

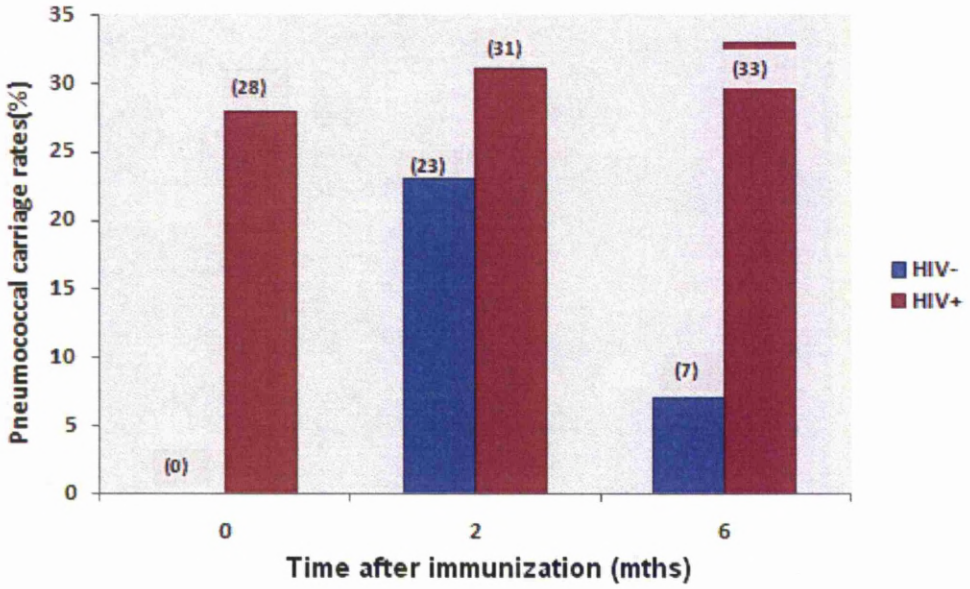


Figure 6.1| Characteristics of pneumococcal carriage in Malawian adults. % pneumococcal nasopharyngeal carriage rates following administration of 7-valent pneumococcal conjugate vaccine (PCV7)

6.3.3 Characteristics of CD4 T cells and CD4 T cell subpopulations in vaccinees

6.3.3a Peripheral blood CD4 T cell counts maintained throughout the study period

The CD4 T cell counts of the HIV positive participants were measured at every study visit to be sure that the HIV-infected participants are immunologically stable during the period of observation and to confirm that the counts are different from those of HIV-uninfected individuals, as shown in previous chapters. At baseline the median CD4 T cell count in HIV-infected individuals (well established on ART) was 376 cells/µl (67-1043). The CD4 T cell counts remained relatively unchanged for the entire period of the study (355 cells/µl [67-992] at 2 months post-vaccination, p=0.6069, W=37.00 and 386 cells/µl [78-848] at 6 months post-vaccination, p=0.1579, W=-88.00) (Figure 6.2). Thus there was little or no reconstitution of CD4 T cells in this group during the study period. As expected, the CD4 T cell counts of HIV-infected study participants were significantly lower compared with non-HIV study participants (p=0.0001, U=43.00, 6 months post-vaccination).

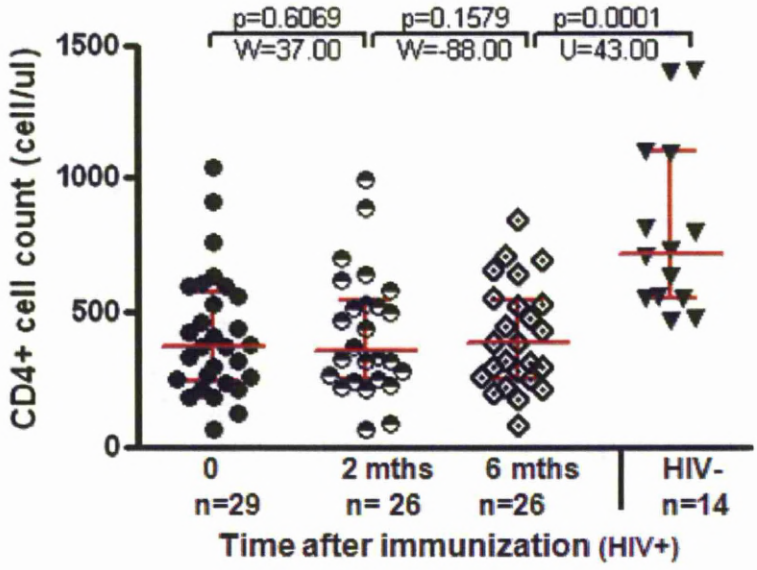


Figure 6.2|Stable peripheral blood CD4 T cell counts in HIV-infected patients (well-established on ART) during the study period a| CD4 T cell counts were measured at baseline (pre-vaccination), 2, 6 months post-vaccination and in HIV-uninfected adults . Red horizontal bars represent median values of CD4 subsets. Statistical significance was analysed using the Wilcoxon matched pairs when comparing CD4+ T cell counts of HIV-infected persons on ART, and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison.

6.3.3b CD4+ naive and memory cells in study participants

The CD4 T cell subsets (naive, central and effector memory) were assessed at the beginning of the study and end of the study to: i) assess the effect of vaccination on CD4+ T cell subsets in HIV-infected individuals on ART and ii) to assess the profile of CD4 T cell subsets in HIV-infected individuals well established on ART and establish whether it is different from that of HIV negative individuals. The proportion of naive and memory (central and effector) in HIV-infected individuals did not change during the study period (Figure 6.3a; naive: p=0.2977, W=-45.00; central memory: p=0.0976, W=-71.00; effector memory: p=0.3438, W=41.00). Similar results were obtained for the proportions of central and effector memory T cells in HIV negative individuals (Figure 6.3b; p=0.3591, W=34.00 and p=0.6257, W=17.00 respectively). The relative size of naive T cells in the non-HIV group was however significantly elevated at 6 months (Figure 6.3b; p=0.0067, W=-92.00). There was no difference in the proportions of naive, central and effector memory T cells between HIV positive and HIV negative participants at 6 months after immunisation (Figure 6.3c; naive: p=0.8808, U=145.0; central memory: p=0.2237, U=113.0; effector memory: p=0.4533, U=127.0). As expected, the absolute counts were significantly lower in HIV positive participants compared to HIV negative study participants (Figure 6.3d; naive: p=0.0251, U=71.00; central memory: p<0.0001, U=18.00; effector memory p=0.0013, U=44.00).

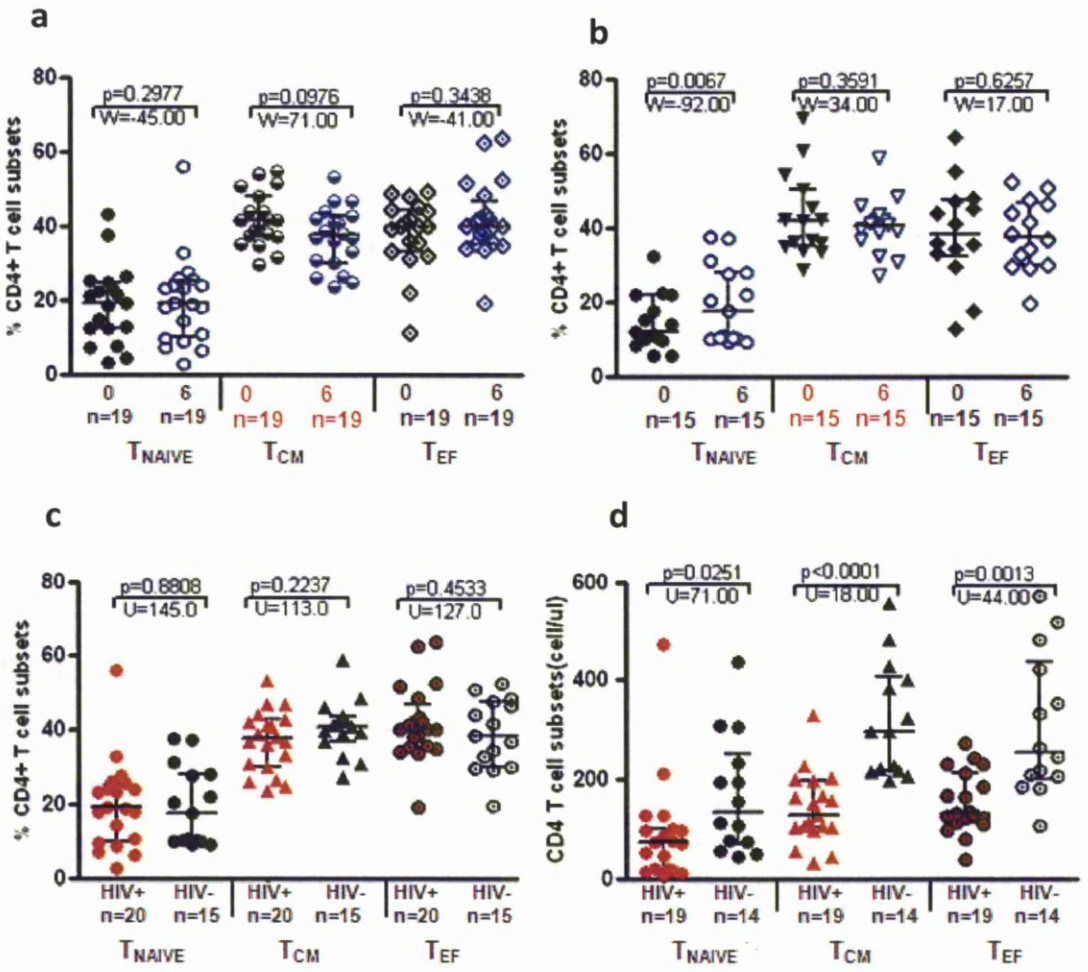


Figure 6.3 | Phenotypic analysis of CD4+ T subsets in peripheral blood. **a|** Proportions of naive, central and effector memory CD4 T cells in peripheral blood of HIV-infected; baseline versus 6 months post-vaccination **b|** Proportions of naive, central and effector memory CD4 T cells in peripheral blood of HIV-uninfected; baseline versus 6 months post-vaccination **c|** Proportions of naive, central and effector memory CD4 T cells in peripheral blood of HIV negative and infected persons 6 months post vaccination (red-HIV-infected and black HIV negative) **d|** Absolute numbers of naive, central and effector memory CD4 T cells in peripheral blood of HIV negative and infected persons 6 months post vaccination. Black horizontal bars represent median values. Differences were calculated using the Wilcoxon matched pairs and the Mann Whitney U test. T_{CM} - central memory, T_{EF} – effector memory.

6.3.4 Functional analysis of protein antigen-specific CD4 T cells after vaccination

6.3.4a Cytokine responses primed by immunization or natural exposure

To characterise cytokine responses primed by immunization or natural exposure, PBMCs from the same human subjects (before and after vaccination) were stimulated overnight with diphtheria (DT), pneumococcal concentrated culture supernatant (D39), and *M. tuberculosis* PPD. The cells were stained intracellularly for IL-2 and IFN-γ. At 0 month point (before vaccination), PBMCs samples from HIV negative study participants stimulated with diphtheria toxoid had few, if any cytokine-producing cells (Figure 6.4a). In comparison, cells treated with pneumococcal proteins (to assess naturally induced immunity), produced IL-2 and IFN-γ individually (Figure 6.4c), while the cells stimulated with PPD produced IL-2 or IFN-γ individually or in combination (Figure 6.4e). Both pneumococcal and PPD-specific responses were maintained throughout the study period.

2 months after the administration the PCV7, cells stimulated with DT were predominantly IL-2 producing (median response 0.05%[interquartile range, 0.00-0.08]) and this response tended to be lower at 6 months after vaccination (median response 0.03%[interquartile range, 0.00-0.0575])(Figure 6.4a). We also observed cells making IFN-γ at the 6 month point. In HIV-infected study participants DT responses were not seen before or after vaccination (Figure 6.4b [median response at 2 month point 0.00% (interquartile range, 0.00-0.0075)]. There was little or no difference in the median responses for both pneumococcal proteins and PPD between HIV positive individuals and HIV negative persons.

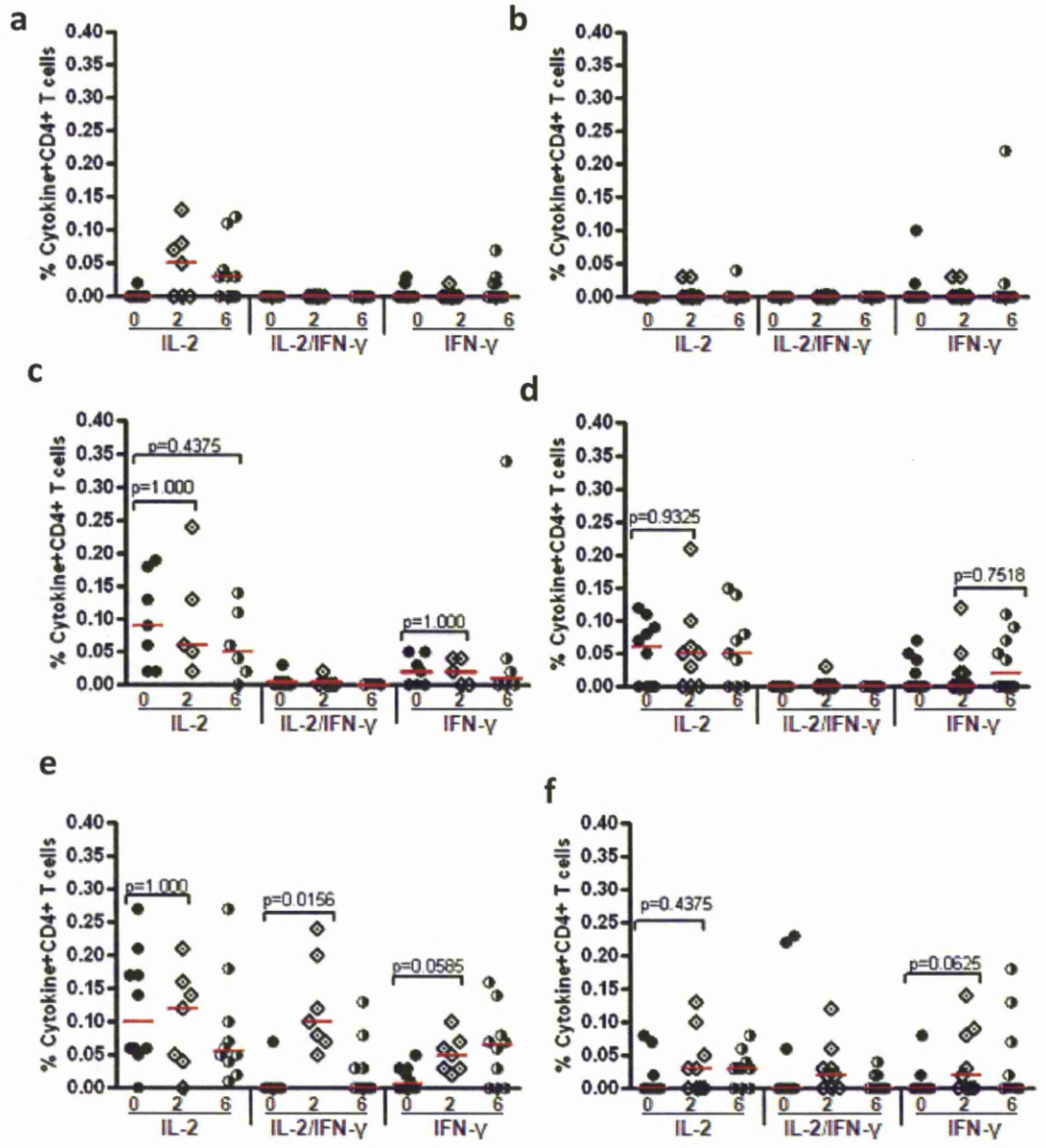


Figure 6.4|Cytokine responses primed by immunization or natural exposure. Cytokine responses to **a|** diphtheria toxoid (DT)-HIV negative vaccinees **b|** DT-HIV positive vaccinees **c|** pneumococcal proteins - HIV negative vaccinees **d|** pneumococcal proteins-HIV positive vaccinees **e|** positive control PPD- HIV negative vaccines **f|**PPD-HIV positive vaccinees. Red horizontal bars represent median values CD4+ T cell responses. Differences were calculated using the Wilcoxon matched pair. 0 – 0 mth point (pre-vaccination). HIV negative (n=9) and HIV positive (n=9-10). 2- 2 mths post-vaccination, 6- 6 mths post-vaccination. IL-2- cells producing IL-2 only, IL-2/IFN-γ- cells producing both IL-2 and IFN-γ and IFN-γ- cells producing IFN-γ only

6.3.4b Poor vaccine (DT)-induced CD154 expression

Activated CD4+ T cells express CD154, providing costimulatory signals to B cells and antigen-presenting cells. CD154 is therefore a central molecule in immune responses. Additionally, CD154 marks a wide array of antigen-responsive CD4 T cells including nearly all cells that express IFN-γ, IL-2 and TNF-α (Chattopadhyay, Yu et al. 2005; Bolton and Roederer 2009). Thus CD154 alone can be used as global indicator of antigen specific CD4+ T cell responses. We therefore tested whether diphtheria toxoid presented to the immune system in the context of a vaccine (PCV7) induces expression of CD154 in CD4+ T cells in HIV negative vaccinees. Additionally, we wanted to assess whether CD154 can be used as a surrogate marker of vaccine-induced responses. DT-specific responses were compared to those induced by natural exposure to the pneumococcus or *M. tuberculosis* PPD. As expected CD4+ T cells stimulated with pneumococcal protein antigens expressed CD154. The proportion of CD4+ T cells expressing CD154 following stimulation with pneumococcal proteins remained unchanged throughout the study period (Figure 6.5b, 0 (pre-vaccination) versus 2 months post vaccination p=0.5807; 2 months versus 6 months post vaccination p=0.7525).

There was poor induction of CD154 on CD4+ T cells in response to diphtheria toxoid following PCV7 vaccination (Figure 6.5c, 0 [pre-vaccination] versus 2 months post vaccination p=0.0863; 2 months versus 6 months post-vaccination p=0.2837), suggesting that at this early time point of vaccination, the vaccine-elicited cells may not provide CD154-mediated costimulation. As previously observed, *M tuberculosis PPD* also induced CD154 expression. There was a non-significant increase in PPD specific responses after 2 months of vaccination (Figure 6.5d, p=0.0515) but these dropped back to the pre-vaccination levels at 6 months post-vaccination (p=0.0313).

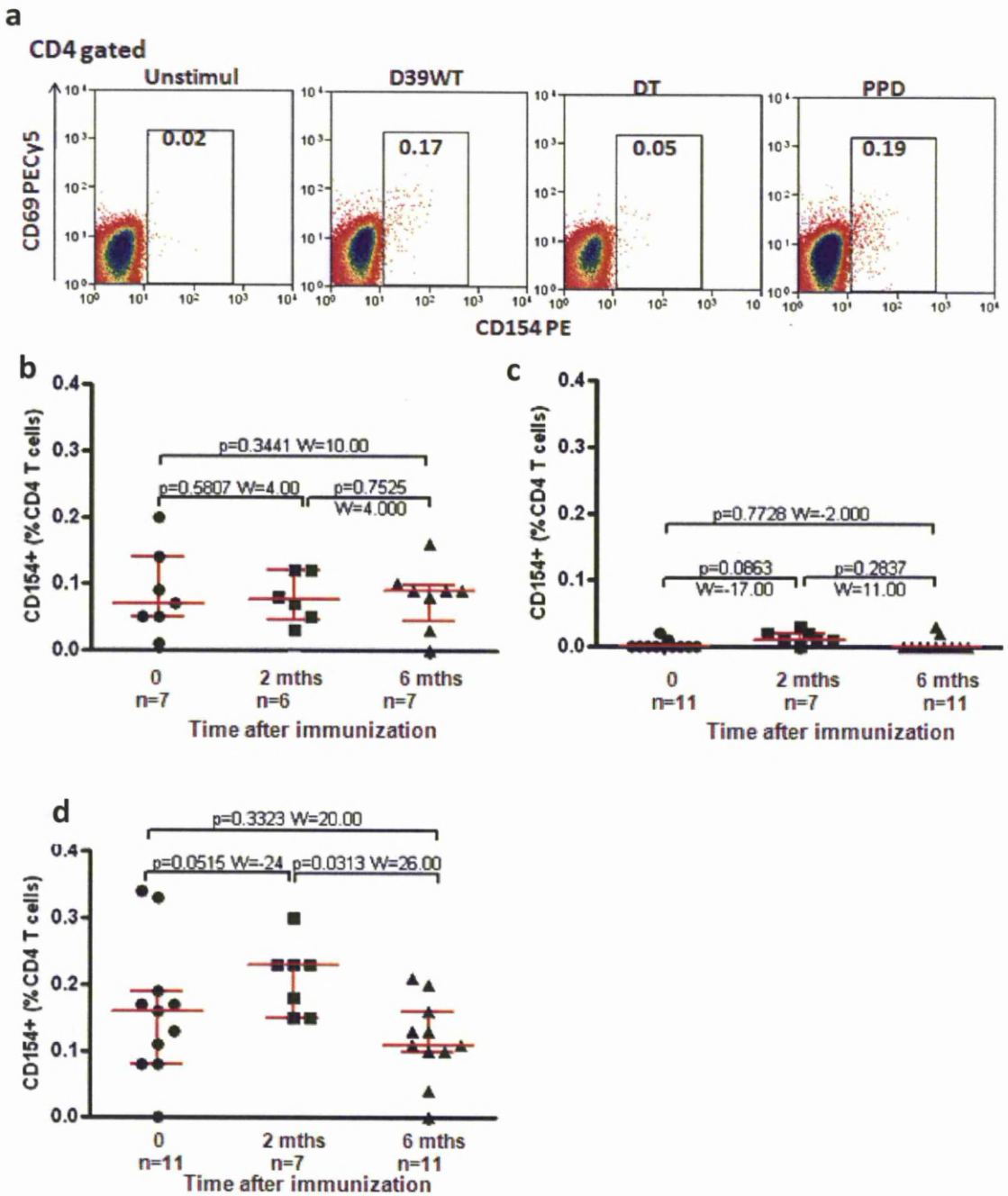


Figure 6.5 | Induction of CD154 (CD40L) on CD4 T cells from HIV negative vaccinees a| Representative plots.The numbers in the gated represent the percentage CD154 expressed on CD4 T cells. Induction of CD154 in response to **b|** pneumoCCS derived from a WT pneumococcal strain (D39WT) **c|** diphtheria toxoid **d|** positive antigen control *M. tuberculosis* PPD. Red horizontal bars represent median values of total responses minus background. Differences were calculated using the Wilcoxon matched pairs.

6.3.5 Characteristics of memory B cells in vaccinees

Memory B cells and memory B cell subsets were assessed at the beginning and end of the study to be sure that the HIV-infected participants are immunologically stable (in the context of memory B cells) during the study period and to confirm that HIV-uninfected and infected are different as described in previous chapters. The percentage of circulating memory B cells in HIV positive individuals at the beginning of the study and at 6 months after vaccination were similar (Figure 6.6a, median 30.13% [11.03–59.80%] and 26.52% [9.660–52.38%], respectively; p=0.7795, W=-16.00). The proportion of memory B cells was slightly lower at the beginning of the study in HIV negative persons (Figure 6.6b, median 53.80% [33.9-70.70%] and 52.00% [37.2-75.00%] respectively; p=0.0195, W=-39.00). At both time points, the percentages of memory B cell were also significantly higher in the HIV negative group than the HIV positive group (Figure 6.6c, 0 months: p=0.0018, U=34.00 and 6 months post immunization: p=0.0004, U=14.00). There was no significant change in the proportion of switched memory B cells during the 6 month period in both HIV positive and HIV negative individuals (p=0·3786, W=-32.00 and 0·1289, W=-27.00 respectively, Figure 6.7a,b). The median frequency of switched memory B cells tended to be lower in HIV positive vaccinees compared to HIV negative vaccines although not statistically significant during the 6 month period(p=0.1068, U=43.00 and p=0.2458, U=51.00 respectively. Figure 6.6c).

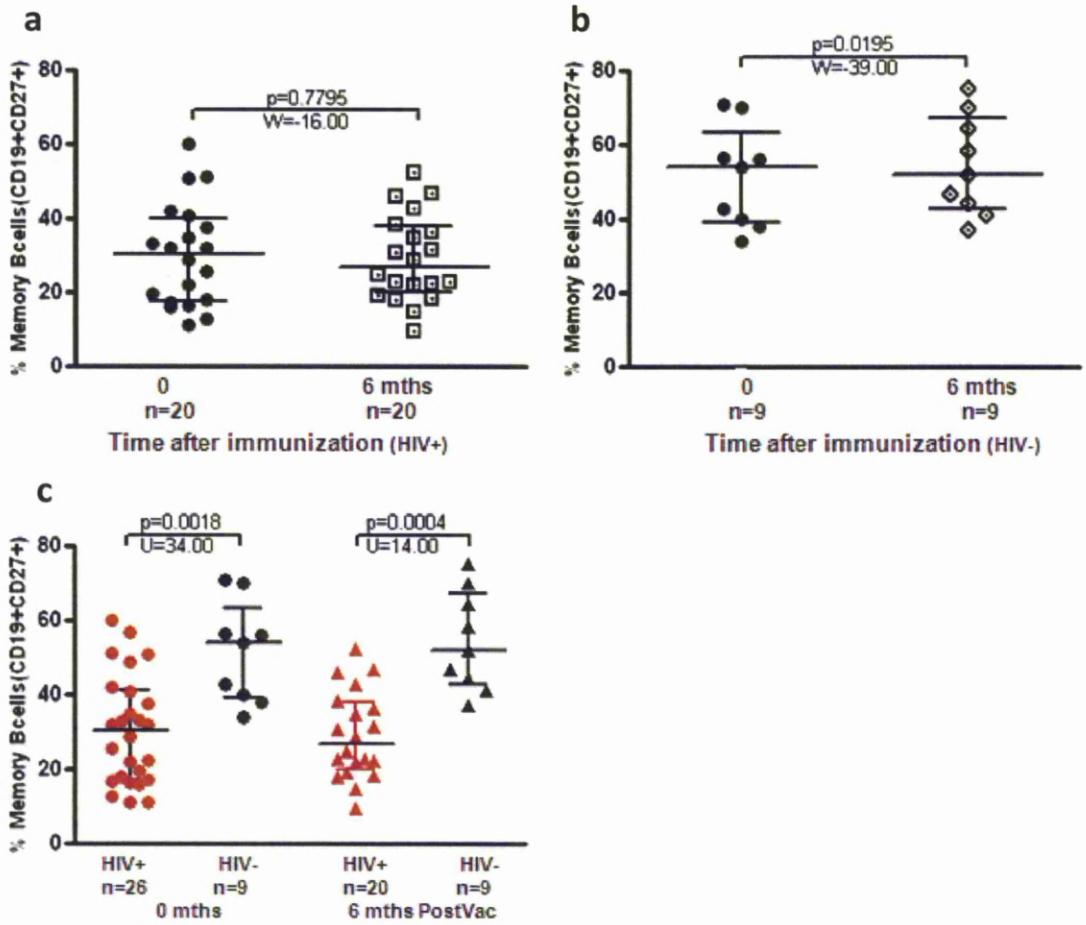


Figure 6.6|Phenotypic analysis of memory B cells in peripheral blood of vaccinees The frequency memory B cells in PB of **a|** HIV positive vaccines **b|** HIV negative vaccinees before and after vaccination and **c|** The frequency total memory B cells in PB of HIV positive and negative vaccinees: a comparison. Black horizontal bars represent median values of memory B cells. Differences were calculated using the Wilcoxon matched pairs and the Mann Whitney U tests.

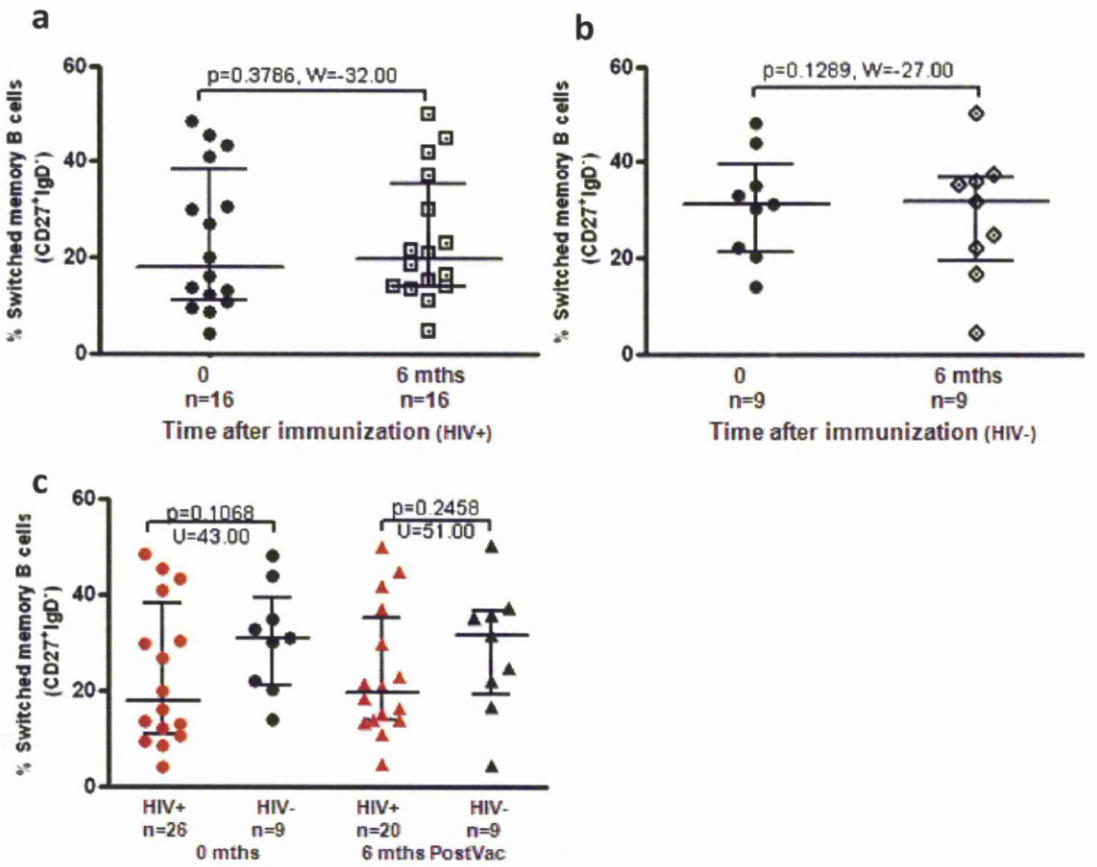


Figure 6.7|Phenotypic analysis of switched memory B cells in peripheral blood of vaccines. Frequency of memory B cells in PB of **a|** HIV positive vaccines **b|** HIV negative vaccinees before and after vaccination and **c|** frequency of switched memory B cells in PB of HIV positive and negative vaccinees: a comparison. Black horizontal bars represent median values of memory B cells. Statistical significance was analysed using the Wilcoxon matched pairs when comparing B cells within a group (HIV-infected persons on their own or HIV-uninfected on their own), and the Mann Whitney U test in the HIV-infected and HIV-uninfected comparison

6.3.6 Functional analysis of memory B cells following vaccination

The function of memory B cells in both HIV negative and positive following administration of the vaccination was assessed by IgG B cell ELISpot. The median number of IgG producing memory B cells to diphtheria toxoid was significantly higher after 2 months of administration of the vaccine compared to baseline levels in both HIV negative and HIV positive vaccinees (Figure 6.8a,b, HIV negative: p=0.0002; HIV positive: p=0.0096). However, 6 months after vaccination, the frequency of diphtheria toxoid specific memory B cells in the HIV positive vaccinees decreased significantly compared to 2 months after vaccination (p=0.0090) and compared well with levels seen at baseline (p=0.4333)(Figure 6.8b). As expected, no post vaccine effect was observed to tetanus toxoid (Figure 6.8c, 2 months post-vaccination: p=0.5934). The median numbers of IgG producing memory B cells to both diphtheria and tetanus toxoid remained consistently higher at all time points in HIV negative vaccinees compared to HIV positive ones (Figure 6.8c,d).

An arbitrary cut-off of 10 antibody secreting cells (ASC) was used to distinguish 'high responders' from 'low responders'. The number or percentage of 'high responders' in response to diphtheria toxoid increased in both HIV negative and HIV positive vaccines following vaccination (Figure 6.9a,b. HIV negative: Baseline 7%, 2 mths 73% and 6 mths 67%; HIV positive: Baseline 3%, 2 mths 21% and 6 mths 12%). There were more 'high responders' in the HIV negative group compared to the HIV positive group. The percentage of 'high responders' was somewhat maintained in the HIV negative group while in the HIV positive group it decreased by nearly 50% 6 months after administration of the vaccine but remained higher than baseline (3%[baseline] versus 12%[6 months post vaccination]).

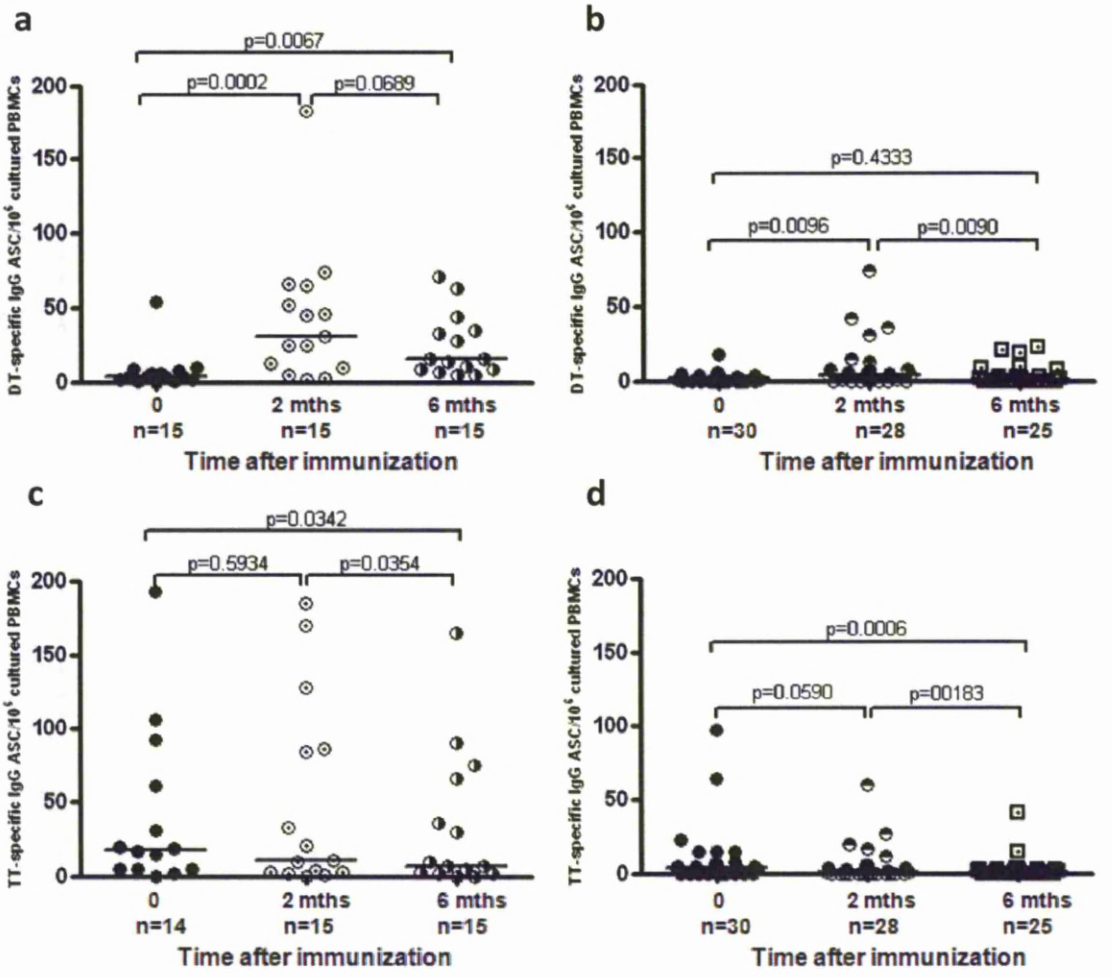


Figure 6.8| PCV7 induces diphtheria toxoid specific IgG memory B cells in the circulation of recently vaccinated adults. Memory B cell responses were expressed as numbers of ASC per million cultured PBMC seeded on the ELISPOT well. **a|** diphtheria toxoid specific responses in HIV negative vaccinees **b|** diphtheria toxoid specific responses in HIV positive vaccinees **c|** tetanus toxoid specific responses in HIV negative vaccinees **d|** tetanus toxoid specific responses in HIV positive vaccines.

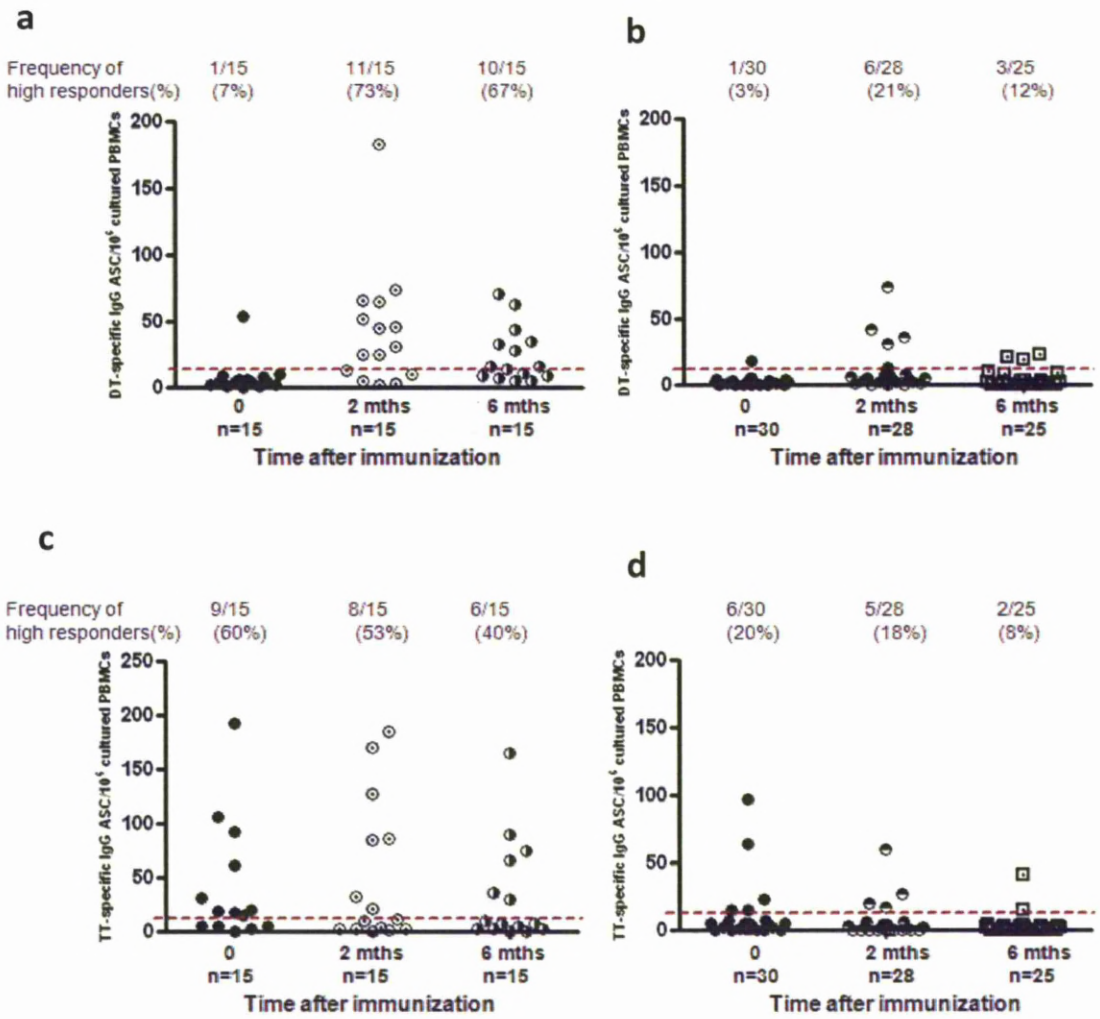


Figure 6.9| PCV7 induces an increased frequency of high responders to diphtheria toxoid. Memory B cell responses were expressed as numbers of ASC per million cultured PBMC seeded on the ELISPOT well. **a|** diphtheria toxoid specific responses in HIV negative vaccinees **b|** diphtheria toxoid specific responses in HIV positive vaccines **c|** tetanus toxoid specific responses in HIV negative vaccinees **d|** tetanus toxoid specific responses in HIV positive vaccines. An arbitrary cut-off (brown dotted line) of 10 ASC was set for antigen specific responses to distinguish 'high responders' from 'low responders'.

6.4 Discussion

Having established in previous chapters that naturally-induced immunity is compromised in HIV-infected adults, this study investigated the impact of HIV infection on immune responses mounted in response to vaccine antigens. Cellular and humoral immune responses to diphtheria toxoid were assessed following PCV7 vaccination in HIV negative and HIV positive Malawian adults well established on ART.

Comparing vaccine elicited and naturally-induced CD4 T cell cytokine responses within the same HIV negative human subjects, we found that CD4 T cell responses to diphtheria toxoid were dominated by IL-2 production. CD4 T cells responses primed by natural exposure (responses to pneumococcal proteins) or a combination of natural and immunization (responses to *M tuberculosis* PPD) comprised IL-2 or IFN-γ individually or a combination of the two. These observations are consistent with studies done elsewhere. A study investigating immune responses to immunization with two well-established licensed vaccines, hepatitis B virus and tetanus, showed that IL-2 was the dominant response (De Rosa, Lu et al. 2004). A similar observation was made by Divekar *et al.* They found that human CD4 T cells primed by diphtheria, tetanus and hepatitis B mostly secreted IL-2 whereas in the same individuals most CD4 T cells initially primed with live pathogens secreted IFN-γ (Divekar, Zaiss et al. 2006). Thus IL-2 may be good surrogate marker of vaccine-induced immune responses.

In HIV-infected vaccinees, DT induced poor cytokine responses while responses to pneumococcal proteins were fairly comparable to those in HIV negative persons. Cytokine responses to PPD were generally lower in HIV-infected vaccinees. Poor responses to the vaccine antigen observed in HIV-infected persons may be partly due to relatively low CD4 T cells counts in this group compared to the non-HIV groups and may also be a reflection of intrinsic defects in the CD4 T cell compartment such as T cell anergy (Hardy, Imami et al. 2003). The fact that responses to vaccine may be poor in HIV –infected including those on ART, brings to the fore the need for the development of efficient vaccination strategies for use in HIV-infected persons.

We also investigated the ability of vaccine (DT) - elicited CD4 T cells to provide T cell help and compared this to responses induced by natural exposure. The expression of the costimulatory molecule CD154 was used as a measure of T cell help. Data presented herein show that CD4 T cells generated naturally can offer CD154 mediated help. This differs from vaccine (DT)-induced responses where CD154 upregulation did not take place, suggesting that CD4 T cells generated immediately after vaccination may not provide CD154-mediated costimulation. Furthermore, this observation suggests CD154 may not be a good early surrogate marker of vaccine-induced responses.

A similar profile of CD154 expression was observed by Chattopadhyay *et al.* after analysis of CD4 T cell responses from 3 individuals with natural CMV infection and 4 individuals who had received a DNA vaccine encoding HIV-envA (Chattopadhyay, Yu et al. 2005). Our results need further investigation with pneumococcal protein-based vaccines (as they become available) and a larger sample size but suggest that there may be heterogeneity in the way protein antigens elicit T-cell help when presented to the immune system in the context of a vaccine and during natural exposure. In our study, we were unable to explore the relationship between CD154 and cytokine expression. However, studies done elsewhere have shown that most of the naturally-elicited cytokine producing cells were capable of CD154-mediated help while cytokine producing CD4 T cells generated immediately after vaccination (and producing only IL-2) were unable to provide CD154-mediated costimulation (Chattopadhyay, Yu et al. 2005).

Analysis of memory B cell to diphtheria toxoid revealed significant differences in numbers of memory B cells to diphtheria toxoid, between pre- and post-vaccination samples and between HIV-infected and HIV-uninfected individuals. Both HIV negative and HIV positive adults had higher frequencies of diphtheria toxoid specific memory B cells detectable 1 month after complete dose of PCV7 vaccination but dropped by 6 months (almost to baseline levels in HIV positive adults).

Diphtheria memory B cell frequencies were generally more robust in HIV negative vaccinees compared to HIV positive vaccinees, consistent with observations made by Malaspina *et al.* that memory B cell responses to T cell dependent antigens are impaired during HIV infection (Malaspina, Moir et al. 2005). Whereas others have shown that immunologic responses to vaccination with T cell dependent antigens are impaired in HIV –infected adults with low CD4+ T cells (Kroon, van Dissel et al. 1995), our data suggest that this impairment is somewhat maintained in individuals well established on ART. Since the CD4 T cell count in the HIV-infected person has not fully recovered, retarded memory B cell DT-specific responses in HIV positive adult on ART may be due to inadequate CD4+ T cell help, or simply low numbers of DT-specific B cell clones. The number of tetanus toxoid (a non-vaccine related control) specific memory B cells was also higher in the HIV negative group compared with the HIV positive group. In this study, we observed DT response (though at minimal levels) before the administration of the vaccine. Natural immunity to diphtheria is however rather unlikely (Nanan, Heinrich et al. 2001) and the responses detected to it were probably residual immunity to previous vaccination. Serology (antibody titres in serum) for the samples obtained during the study is yet to be performed.

In summary, we have shown that IL-2 may be good early indicator or surrogate marker of vaccine-induced immune response and that there may be some heterogeneity in the way protein antigens elicit T-cell help. Data presented in this chapter also show that cellular and humoral responses to protein vaccine antigens are compromised and most likely short-lived in HIV-infected persons including those stable on ART. This calls for development of efficient vaccination strategies for use in HIV-infected persons. It will also be important to evaluate the longevity of T and B cell immunity to novel pneumococcal protein vaccines in both HIV positive and negative as they become available.

CHAPTER 7 Final Discussion

7.1 Introduction

Streptococcus pneumoniae (the pneumococcus) is a bacterial commensal that colonizes the mucosal surface of the human nasopharynx and only rarely causes disease in healthy adults (Obaro and Adegbola 2002). Pneumococcal proteins are major determinants of this adaptation, facilitating both colonisation and invasion through host cell attachment, toxicity and inflammation (Ogunniyi, Lemessurier et al. 2007; Giefing, Meinke et al. 2008). Our research group has previously demonstrated the acquisition of natural T cell memory responses to pneumococcal protein antigens in an adult population in an area of high pneumococcal carriage and disease (Mureithi, Finn et al. 2009). The study revealed that memory T-cell immunity to pneumococcal protein antigens consisted of both effector memory phenotypes (T_{EM}) marked by $CD4^{+}CD45RO^{+}CCR7^{-}$ T-cells which are implicated in pathogen clearance and long term resting central memory T-cells (T_{CM}), marked by the expression of $CD4^{+}$ $CD45RO^{+}CCR7^{+}$ thought to be responsible for immune surveillance (Sallusto, Lenig et al. 1999; Geginat, Sallusto et al. 2001).

Although it is well established that HIV infection significantly increases the risk of invasive pneumococcal disease, little is known about the effect of HIV infection on the pneumococcal antigen-specific immunity described above. This is the first study to extensively assess the influence of HIV on natural- induced pneumococcal immunity in an area of high pneumococcal carriage, invasive disease and HIV infection. This thesis provides evidence that: (i) naturally induced pneumococcal-specific CD4+ T cell responses and the CD154 pathway are compromised in asymptomatic HIV-infected adults; (ii) in the context of persistent carriage, ART results in some regeneration of T cell responses to the pneumococcus by 6 months; and (iii) immune responses against protein antigens presented to the immune system in the context of a vaccine may be compromised in HIV-infected adults including those well-established on ART.

These findings have raised several interesting questions about natural immunity to this commonly carried commensal and its reconstitution, the timing of ART, the possibility of using vaccines to provide additional protection to individuals starting ART with low CD4 T cell count and the durability and robustness of vaccine-induced immune memory in HIV-infected individuals including those on ART. These questions will be addressed in this chapter.

7.1.1 Impaired CD4 T cell functional responses to *Streptococcus pneumoniae* in asymptomatic HIV-infected Malawian adults

7.1.1.1 Phenotypic changes in asymptomatic HIV infected adults

In chapter 4, we carried out a detailed assessment of CD4 T cell subsets in both HIV-infected and uninfected individuals. In addition to loss of CD4 T cells in HIV infection (i) circulating central memory (T_{CM}) and naive (T_N) CD4+ T cells were preferentially lost in asymptomatic HIV infection; and (ii) there was overrepresentation of senescent and regulatory T cells in HIV-infected individuals

There is now extensive literature to indicate that loss of CD4+ T cell subsets during HIV infection may be due to selective infection and killing of CD4+ T cells and constant recruitment of cells from one pool to another pool (Hazenberg, Hamann et al. 2000; McCune 2001; Grossman, Meier-Schellersheim et al. 2002; Silvestri and Feinberg 2003; Stevenson 2003). HIV replicates preferentially in memory CD4+ T cells expressing CCR5 (a co-receptor used by HIV to infect CD4+ T cells), leading to a depletion of these cells, something that is particularly rapid and pronounced in the gut (Brenchley, Schacker et al. 2004). Conversely, HIV infection is characterised by chronic immune activation and because of this activation there is constant recruitment of CD4 T cells from the naive and central memory pools into the effector pool, creating a strain on the CD4 T cell homeostasis (Hazenberg, Hamann et al. 2000; McCune 2001; Grossman, Meier-Schellersheim et al. 2002).

Early loss of naive T cells in HIV infection is partly due to abnormal thymic function. Maintenance of CD4 T cell homeostasis depends partly on the ability of the thymus to continuously export new naive T cells,- because naive T cells cannot be regenerated by peripheral mechanisms (Mackall and Gress 1997; Berzins, Uldrich et al. 2002; Ge, Hu et al. 2002). HIV infection however compromises thymic export which results in poor replenishment of naive T cells in the periphery (Douek, McFarland et al. 1998; Hazra and Mackall 2005; Sempowski, Hicks et al. 2005). It is not clear what impact early depletion of naive T cells (mostly as a result of abrogated thymic export) has on the control of infections or pathogens. Nonetheless, studies done using animal models suggest that continued replenishment with cells from the thymus is required to maintain efficient gut mucosal defence (Bourgeois, Hao et al. 2008). Furthermore, naive CD4+ T cells are very sensitive to activation and for that reason HIV-associated chronic immune activation may cripple activated CD4 T cell responses against pathogens, such that only export of new naive T cells by the thymus can ensure sustained control of infections (Bourgeois, Hao et al. 2008).

As observed in chapter 4, there was an increase in the proportion of effector memory T cells in asymptomatic HIV-infected individuals. In contrast, the absolute number was not significantly changed. The expansion of effector memory cells was however accompanied by a decrease in the fraction and absolute number of central memory CD4 T cells, suggesting that the expansion of effector memory T cells was due to a selective absolute loss of the central memory cells. Using a different phenotypic classification of CD4+ T cell subsets based on the expression of CD127 and CD25 (receptors for IL-7 and IL-2 respectively), Dunham *et al.* found that the relative expansion of the CD4+ CD127-CD25- subset (effector memory CD4+ T cells), was due to a selective absolute loss of the CD127-CD25$^{low/-}$ subset (naive and central memory) (Dunham, Cervasi et al. 2008). Consistent with our observation, they noted that the proportional expansion of effector memory CD4+ T cells correlated with decreased CD4+ T cell levels and the levels of immune activation (marker of disease progression). Dunham *et al.* therefore concluded that the expansion of effector memory T cells reflected both as a determinant and as a consequence, the HIV-associated chronic immune activation.

As noted in the data presented in chapter 4 and other studies done elsewhere, regulatory T cells expand as HIV disease progresses. A number of explanations have been given in an attempt to explain Tregs expansion among HIV-infected persons. Among these are: either the Tregs are not depleted during HIV infection or that they are expanded as an attempt by the immune system to slow down disease progression. Studies show that Tregs are just as susceptible to HIV infection as memory T cells (Oswald-Richter, Grill et al. 2004). However, it has been proposed that immune activation drives non Tregs (CD4+CD25-FOXP3-) (upon activation) to convert to Tregs (CD4+CD25+FoxP3+) (Chen, Jin et al. 2003; Walker, Kasprowicz et al. 2003). A chronic state of activation (as seen during HIV infection) thus changes the rate at which Tregs are produced while providing a new avenue of cell loss of other T cell subsets.

An important and unanswered question is whether Tregs play a protective role or detrimental role during HIV infection. Tregs have been implicated in the suppression of HIV-specific T cell responses such that Tregs cell depletion enhances cytokine production and proliferation by T cells *in vitro* (Aandahl, Michaelsson et al. 2004; Kinter, Hennessey et al. 2004; Weiss, Donkova-Petrini et al. 2004; Kinter, Horak et al. 2007). For this reason, some argue that an increase of Tregs may impair the immune control of HIV-1 infection. Our observations and also those from elsewhere (Cao, Jamieson et al. 2009), show that expansion of Tregs is associated with HIV disease progression suggesting that the expansion of Tregs relative to other CD4+ T cell subsets may result in an increased suppressor-to helper ratio, leading to suppressed T cell immune responses to HIV-1 and other pathogens including *S. pneumoniae* and rapid progression to AIDS.

Recently, an analyses of *in vitro* suppressive capacity of CD25+ regulatory T cells isolated during chronic SIVmac 251 infection in cynomolgus macaques showed that the suppressive capacity of Tregs was associated with high CD4 T cell counts (Karlsson, Malleret et al. 2011) suggesting that in advanced HIV/SIV disease/infection (i.e. in cases of low CD4 T cell counts), the suppressive capacity of the Tregs may be impaired. Thus, in advanced HIV disease, Tregs may have little to do with suppressed T cell immune responses to pathogens. This however will have to be confirmed in human studies.

One way of to investigate the suppressive capacity of Tregs at different stages of HIV disease would be to recruit HIV-infected individuals at different stages of HIV infection and collect peripheral blood. PBMCs would be isolated and split into two portions. Then Tregs would be depleted from one portion of the PBMCs. Thereafter, the cells whether depleted of Tregs or not will be stimulated with antigens to determine whether depletion of Tregs results in increased antigen-specific responses.

7.1.1.2 Compromised pneumococcal antigen-specific CD4 T cell responses in asymptomatic HIV infected adults

Characterisation of the functional properties of pneumococcal antigen-specific CD4+ T cells in HIV-infected Malawian adults revealed that CD4+ T cell responses were either compromised (IFN-γ effector responses, proliferative capacity and CD154 pathway) or altered (IFN-γ/IL-10) in HIV infection (chapter 5).

Several mechanisms may explain the relatively poor effector memory responses to pneumococcal proteins in HIV-infected persons. A simplistic explanation would be that the poor effector responses are due to low numbers of pneumococcal-specific T cell clones, a result of declining CD4 T cell counts. But poor pathogen-specific responses were observed even in individuals with relatively good CD4 T cell count (CD4 T cell count >500 cells/μl). Therefore, poor effector memory responses may in part be caused by poor sustenance of the effector memory T cell population during HIV infection. The effector memory T cell population is normally sustained through antigen-dependent and antigen-independent pathways (Geginat, Sallusto et al. 2001). It is unlikely however that poor pneumococcal-specific CD4 T cell responses observed in HIV-infected persons was caused by a lack of antigen-dependent stimulation because of the high pneumococcal carriage rates in our study population. High pneumococcal carriage rates should help maintain the frequencies of effector memory T cells.

Central memory T cells can under the influence of cytokines derived from dendritic cells (antigen-independent pathway) proliferate leading to the generation of effector memory T cells. Among the cytokines involved in this pathway are IL-15 (Geginat, Sallusto et al. 2001) and IL-23 (Brombacher, Kastelein et al. 2003). It has been shown that the generation of IL-23 by adherent cell fraction of PBMC (monocytes) is impaired in HIV-infected persons and that this was associated with decreased production of IFN-γ by T cells (Lee, French et al. 2004). The authors speculate that these poor IFN-γ effector responses reflect a failure of the antigen-independent pathway to generate effector T cells from a pool of central memory T cells. Similarly, impaired proliferative capacity may be due to lack of sustained antigen-independent maintenance of central memory T cells. In the study described in chapter 4, culture supernatants were not analysed for IL-15 and IL-23. Therefore we were not in position to confirm whether secretion of IL-15 and IL-23 in response to pathogens is impaired in HIV-infected persons and by extension whether poor IFN-γ responses (in response to pathogens) reflect a failure of the antigen-independent pathway in HIV positive persons.

Poor proliferative capacity may also be a reflection of T cell functional impairment in the form of T cell anergy (Beverly, Kang et al. 1992; Hardy, Imami et al. 2003) . Studies have shown that in HIV-infected persons where proliferative responses are impaired, the proliferative responses could be induced with high concentration of IL-2 (Hardy, Imami et al. 2003). On the other hand, IL-2 has been shown to reverse anergic phenotype (Beverly, Kang et al. 1992). Taken together, these observations suggest that the poor proliferative responses observed in chapter 4 may be a result of T cell anergy.

In this thesis, we were able to optimise and adapt a novel assay to assess the CD154 pathway in CD4+ T cells and subsequently used the assay to assess the effect of HIV-infection in pneumococcal antigen- induced CD154 expression. Activated CD4+ T cells express CD154, providing costimulatory signals to B cells and antigen-presenting cells (van Kooten and Banchereau 2000; Ma and Clark 2009). CD154 is therefore a central molecule in immune responses. As noted in chapter 4, pneumococcal –specific induction of CD154 on activated CD4+ T cells was impaired during HIV-infection.

It could be hypothesised that the impaired induction of CD154 is caused by a selective loss of a CD4+ T subset capable of expressing of CD154 upon activation since HIV infection is associated with a progressive decline of CD4+ T cells and subsets. However data presented here and elsewhere (Subauste, Wessendarp et al. 2004) suggest a specific defect in the regulation of the induction of CD154.

The data presented in chapter 4 show that CD154 expression was impaired even in individuals with relatively good CD4 T cell counts (CD4 cell counts above 350 cells/μl) and by extension relatively intact immunity. Using concentrations of CD4+ T cells and monocytes that are similar to those of HIV negative persons(i.e. same ratio of CD4 to monocytes as in HIV-uninfected individuals), Subauste *et al.*, observed that the expression of CD154 in response to *C. albicans* (an extracellular pathogen) was impaired in HIV-infected patients (Subauste, Wessendarp et al. 2004) clearly indicating that the defective induction of CD154 on activated CD4+ T cells goes beyond numeric loss of CD4+ T cells.

As for the mechanism(s) underlying CD154 dysregulation in HIV, *in vitro* studies suggest that interactions between HIV envelope glycoproteins and the CD4 receptor may play a role. Studies show that HIV-1 envelope proteins can cross-link CD4 molecules on the surface of the cell, and inhibit up-regulation of CD154 (Chirmule, McCloskey et al. 1995; Marschner, Hunig et al. 2002; Zhang, Fichtenbaum et al. 2004; Rutjens, Vermeulen et al. 2008). Consistent with data from elsewhere, preliminary data from this laboratory showed that cross-linking of CD4 with mAb inhibit up-regulation of CD154 in CD4+ T cells (Appendix II).

There are several ways defective CD154 signalling could increase susceptibility to infections. These include: decreased production of IL-12 and IFN-γ (CD154 is crucial for optimal in *vitro* production of IL-12 and/or IFN-γ in response to a number of pathogens), impaired macrophage activation, defective priming of CD4+ and CD8+ T cells and an impaired humoral response. Analysis of cytokines in culture supernatants in this laboratory showed no difference in IL-12 concentration between HIV-positive and negative individuals (results not shown).

A number of studies done in mice and humanised mice, however, have shown that CD154 plays a role in the generation of antibodies to pneumococcal proteins and capsular polysaccharides (Hwang, Nahm et al. 2000; Jeurissen, Billiau et al. 2006; Moens, Wuyts et al. 2008). Additionally, neutralization of CD154 has been shown to suppress antibody responses to pneumococcal protein (PspA) (Hwang, Nahm et al. 2000). This suggests that impaired CD154 expression may affect antibody response to pneumococcal proteins and results in poor control of IPD in HIV-infected persons.

7.1.1.3 Implications for clinical management

WHO recommends that ART should be initiated before CD4 T cell count falls below 350 cells/μl regardless of symptoms (WHO 2009). Interestingly, our data show that some antigen-specific CD4 T cell responses are impaired even at CD4 T cell count much higher than 350 cells/μl. Therefore, our findings support WHO recommendations and further suggest use of an even higher CD4 T cell count as a threshold for initiation of therapy. This is consistent with suggestions made elsewhere (Robbins, Spritzler et al. 2009) that ART should be initiated at a threshold of 350 cells/μl or higher CD4+ T cell count at which the CD4+ naive cell populations and naive-memory cell ratios are more likely to be intact. In their study, Robbins et al. observed that CD4+ naive-memory cell ratios were lower among patients with CD4 T cell count <350 cells/μl and this deficit persisted even after nearly 3 years of ART ((Robbins, Spritzler et al. 2009). It is however doubtful if WHO recommendations can be fully implemented in resource poor setting (at least in the short term) for a variety of social and infrastructural reasons such as poorly resourced health centres especially in rural areas and shortage of well-trained health personnel (Van Damme 2006; Libamba, Makombe et al. 2007; USAID 2010).

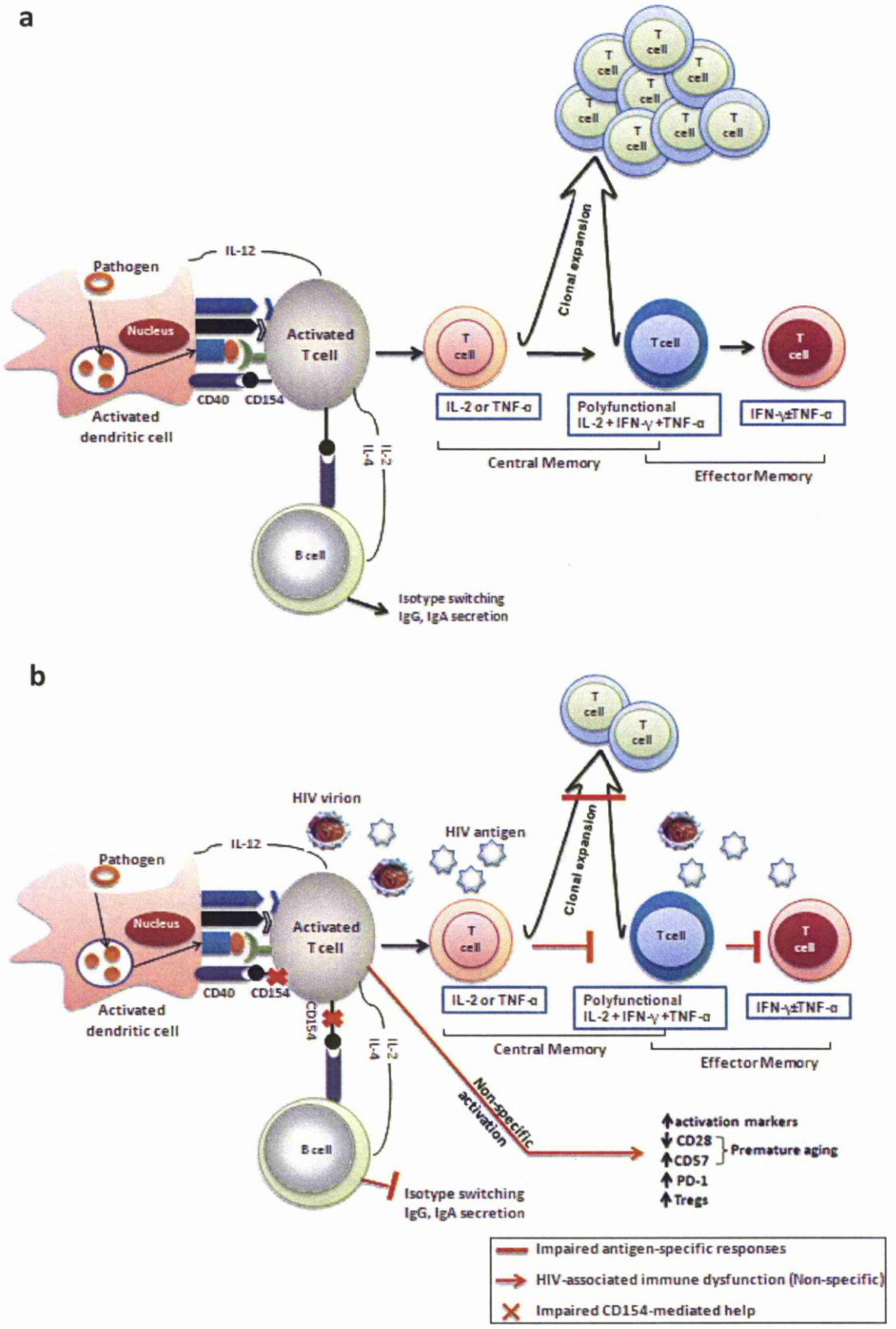
Having demonstrated that pneumococcal-specific immune responses are compromised in asymptomatic HIV-infected adults, it was important to determine whether pneumococcal immunity can be regenerated during ART.

7.1.2 Immune responses to *Streptococcus pneumoniae* following initiation of antiretroviral therapy

7.1.2.1 Regeneration of T cell responses to S. pneumoniae

Data presented in chapter 5 showed that by 6 months ART and in addition to increases in CD4 T cell counts and viral load suppression there was some regeneration of pneumococcal antigen-specific CD4+ T cell responses. These include effector memory responses (*ex vivo* IFN-γ ELISpot responses), ability to proliferate (central memory responses) and ability to produce simultaneously multiple cytokines (polyfunctional capacity) (illustrated in Figure 7.1). However, there was little or no regeneration of either PPD or Flu-specific CD4 T cell responses after 6 months of ART.

There are several hypothetical explanations for the reconstitution of pneumococcal antigen-specific CD4+ T cell responses. As stated above, diminished proliferative responses observed in treatment naive HIV infected individuals may have been due either to very low antigen-specific clone numbers or T cell anergy. The recovery of proliferative responses following weeks of antiretroviral therapy may be a result of reversed T cell anergy (Beverly, Kang et al. 1992; Meyaard, Schuitemaker et al. 1993; Hardy, Imami et al. 2003). Additionally, the reconstitution of central and effector memory responses may reflect the regeneration of the antigen-independent pathway and consequently the capacity to maintain a pool of central memory T cells (Geginat, Sallusto et al. 2001; Lee, French et al. 2004). However, the responses in HIV positive individuals were still lower compared to HIV-negative persons at 6 months ART, suggesting that the regeneration of antigen-independent pathway was incomplete in this group. Another possible explanation for the rejuvenation of an immune response in the periphery in early stages of therapy may be redistribution of sequestered cells (from lymphoid tissues) rather than *de novo* synthesis (Evans, Bonnez et al. 1998; Bucy, Hockett et al. 1999).


a
T cell
Clonal expansion
Pathogen
IL-12
Nucleus
Activated T cell
Activated dendritic cell
CD40
CD154
IL-4
IL-2
B cell
Isotype switching
IgG, IgA secretion
IL-2 or TNF-α
Polyfunctional IL-2 + IFN-γ +TNF-α
IFN-γ±TNF-α
Central Memory
Effector Memory
b
HIV virion
HIV antigen
Non-specific activation
↑activation markers
↓CD28
↑CD57
Premature aging
↑PD-1
↑Tregs
Impaired antigen-specific responses
HIV-associated immune dysfunction (Non-specific)
Impaired CD154-mediated help

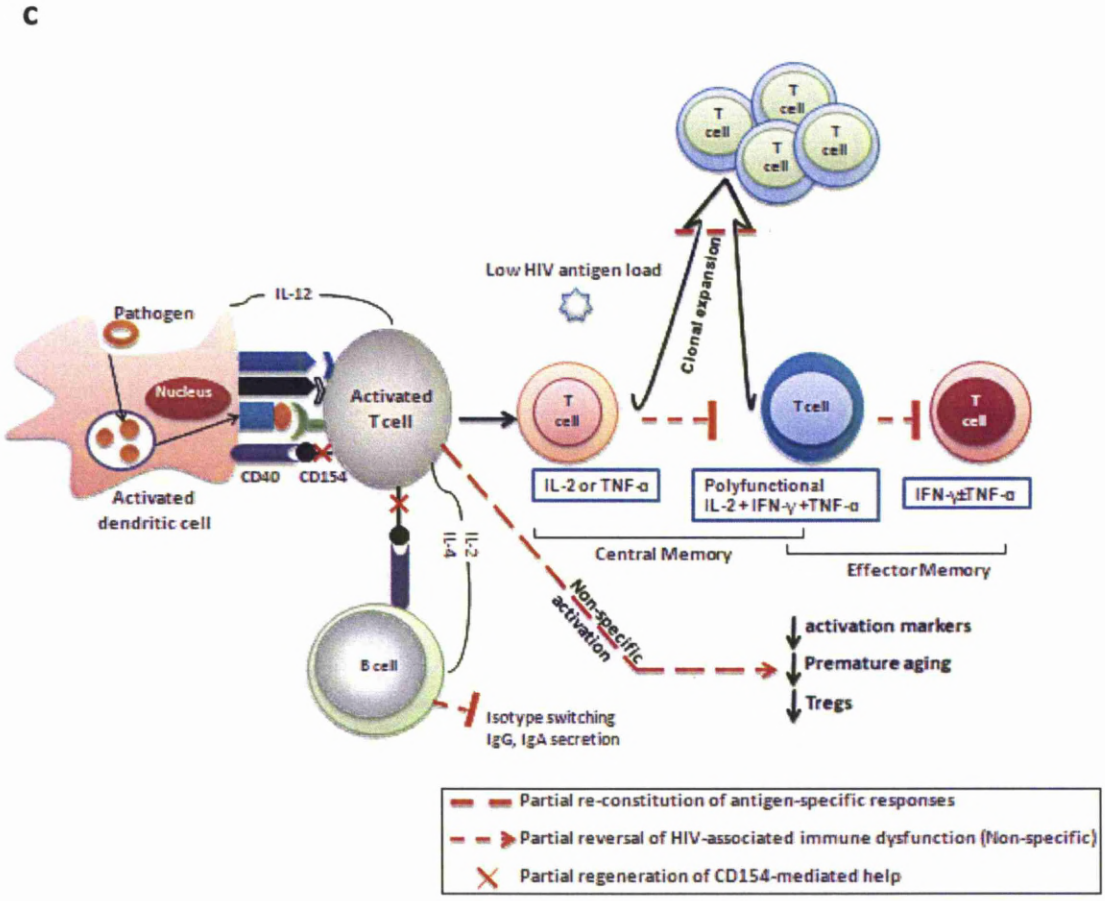


Figure 7.1 | Schematic summary of the influence of HIV infection and ART on antigen-specific responses and immunity in general (peripheral blood) a| On encounter with antigen, T cells proliferate and acquire effector function. Activated cells also acquire ability to provide T cell help (by expressing CD154) to antigen presenting cells and B cells **b|** HIV infection leads to poor antigen-specific CD4 T cell responses (e.g. poor proliferation and cytokine responses), CD4+ T cell help (CD154 expression) to antigen-presenting cells and B cells and B cell responses. HIV infection also leads to premature aging, persistent activation and high proportion of Tregs **c|** Initiation ART leads to some regeneration of antigen-specific CD4 T cell responses, CD4 T cell help, viral load suppression, reduced premature aging and activation.

The fact that T cell responses to pneumococcal antigens, recovered quicker than PPD or flu specific responses may be a reflection of ongoing or persistent encounter of the immune system with pneumococcal antigens (because of high pneumococcal carriage) which help preserve and then regenerate a pool of pneumococcal specific T cell clones during chronic HIV infection and then upon initiation of therapy. It will be difficult to prove this in a setting like Malawi, where carriage rates are very high and as such there is a high prevalence of pneumococcal antigens. Thus, there is a persistent encounter of the immune system with pneumococcal antigens. However, this could possibly be tested in a setting of low nasopharyngeal carriage rate and low antigen prevalence.

As noted in the data presented in chapter 5, there is also some restoration of CD154 expression on CD4+ T cells in response to pneumococcal antigen following initiation of ART. As indicated above, studies on CD154 expression during HIV infection implicated cross-linking of CD4 by HIV envelope glycoproteins in impairment of CD154 expression in HIV-infected individuals (Zhang, Fichtenbaum et al. 2004). Additionally, studies also showed that impaired defective CD154 expression was more prevalent in subjects with detectable plasma viral load (Zhang, Fichtenbaum et al. 2004; Subauste, Subauste et al. 2007). These observations provide a potential explanation for improved CD154 expression following initiation of ART. In our study and at 6 months ART, the participants exhibited viral suppression. Suppression of viral replication means less viral proteins are released into circulation to cross-link CD4 receptors, leading to improved CD154 expression in individuals on ART.

7.1.2.2 No restoration of B cell responses to S. pneumoniae

As shown in chapter 5, ART did not restore IgM memory B cell percentages or numbers. This may partly explain why HIV-infected individuals on ART are still at an increased risk of invasive pneumococcal disease compared to HIV-uninfected persons. In fact, patients with reduced IgM memory B cells counts have been shown to have impaired pneumococcal IgM antibody responses (IgM memory B cell responses are more directed to polysaccharide) post vaccination compared with controls (Hart, Steel et al. 2007).

The importance of IgM memory B cells in host protection against pneumococcal infection have been demonstrated in several patient groups such as children under 2 years of age, the elderly or patients with congenital asplenia. Reduced or absence of IgM memory B cells in these patient groups is associated with increased risk of invasive pneumococcal infection (Kruetzmann, Rosado et al. 2003; Shi, Yamazaki et al. 2005).

Additionally, lack of IgM memory B cells in patients with common variable immunodeficiency (CVID) and asplenic individuals has been associated with increased susceptibility to pneumococcal infections (Kruetzmann, Rosado et al. 2003; Carsetti, Rosado et al. 2004). In a study by Hart *et al.* the reduction in IgM memory B cells in individuals on ART was similar to that seen in patients with CVID (Hart, Steel et al. 2007). Reduced IgM memory B cells in HIV-infected individuals probably explains why the 23-valent pneumococcal polysaccharide vaccine has suboptimal activity in HIV-infected adults and is not recommended for use in Africa (WHO 2008c; French, Gordon et al. 2010).

Although the difference in switched memory B cells between HIV-infected individuals on ART and HIV-uninfected persons was not pronounced, responses to pneumococcal protein CbpA were significantly lower in the HIV-infected on ART. This suggests that HIV-associated immune dysfunction goes beyond the numeric loss of memory B cells. Possible explanations for persistently impaired CbpA-specific responses are that at 6 months ART, therapy had not fully reversed either the intrinsic defects in the B cell response and/ or that persistent defects in the CD4 T cell compartment compromised the ability of CD4 T cells to provide adequate help to B cells (Moir, Ogwaro et al. 2003; Subauste, Subauste et al. 2007).

The fact that IgM memory B cells (whose responses are directed towards polysaccharides) are reduced and antibody responses to pneumococcal proteins are impaired suggests poor responses from both IgM and switched memory B cells contribute to increased risk of invasive pneumococcal infection observed in HIV-infected individuals ART.

7.1.2.3 Implications for clinical management

Although, there was some regeneration of T cell responses, the responses in HIV-infected on ART were still significantly lower compared to controls at 6 months ART, indicating that additional protection may be required perhaps in the form of vaccination. The World Health Organisation guidelines recommend ART initiation when CD4 counts drop below 350 cells/μl regardless of symptoms (WHO 2009). Our data suggest that this may be crucial in the context of retaining adequate response to pneumococcal protein antigens and reduce the risk of IPD in HIV-infected persons on ART.

7.1.3 Immune responses to protein vaccine antigens in HIV-infected Malawian adults on antiretroviral therapy

This part of the thesis investigated immunological memory mounted in response to vaccine antigens in HIV-infected Malawian adults well established on ART. Study participants were drawn from the placebo arm of a recently completed double-blind, randomised, placebo-controlled 7-valent pneumococcal conjugate vaccine trial in HIV-infected and uninfected Malawian adults (French, Gordon et al. 2010). The vaccine trial showed a 74% clinical efficacy for PCV7 in preventing pneumococcal disease caused by one of the vaccine serotypes and a vaccine related serotype 6A in HIV-infected Malawian adults. For the study described in chapter 6, diphtheria toxoid (DT) [carrier protein in the 7-valent pneumococcal conjugate vaccine (PCV7)] was used as a model vaccine antigen.

7.1.3.1 Poor CD4 T cell responses to diphtheria toxoid in HIV-infected adults on ART

In HIV-uninfected, CD4 T cells primed by vaccination with diphtheria toxoid mostly secreted IL-2 in response to diphtheria toxoid whereas in the same individuals most CD4 T cells primed by natural exposure to *S. pneumoniae* secreted IL-2 or IFN-γ individually or in combination in response to *M. tuberculosis* PPD. The fact that IL-2 producing CD4 T cell dominated the vaccine-induced responses suggests that IL-2 may be an early indicator of vaccine-induced responses. Little or no DT responses were detected in HIV-infected individuals on ART. Thus even in individuals well established on ART vaccine T cell responses are compromised. Naturally-induced responses were detected in HIV-infected persons on ART but these were relatively lower than those of HIV negative individuals.

It is not clear why IL-2 is a dominant response for 'diphtheria toxoid' immunization. However, studies done in mice have shown that CD4 T cells that produce IFN-γ are short-lived *in vitro* and *in vivo* and only cells that do not produce IFN-γ efficiently developed into long term memory cells (Wu, Kirman et al. 2002; De Rosa, Lu et al. 2004). Therefore, the fact that it is the IL-2 producing cells that become memory cells may explain the dominance of CD4 T cells that produce IL-2 after 'DT' vaccination.

Studies suggest that vaccine-induced IL-2 only producing cells are uncommitted and under the right polarizing conditions can differentiate into Th1 or Th2 phenotypes (Divekar, Zaiss et al. 2006). This may have implications for pneumococcal protein vaccines especially in HIV-infected persons. If pneumococcal protein vaccines are to induce strong effector T responses additional signals (probably in the form of potent adjuvant) may be required to push the primed but uncommitted CD4 T cells into efficient effectors.

The fact that DT responses were less robust in HIV-infected group emphasises the need to develop efficient vaccination strategies for use in HIV-infected persons including those on ART. This observation also raises the possibility that although current vaccination strategies may offer short-term protection, additional booster doses may be required to achieve long lasting immune responses.

While pneumococcal proteins and PPD induced expression of CD154 in CD4+ T cells, diphtheria toxoid did not induce CD154 expression, even in healthy individuals, suggesting that CD154 may not be good early correlate of vaccine efficacy or an early indicator of vaccine-induced responses. This observation also suggests heterogeneity in the way protein antigens elicit T-cell help when presented to the immune system in the context of a vaccine and during natural exposure.

7.1.3.2 Poor B cell responses to diphtheria toxoid in HIV-infected adults on ART

Memory B cells are central to the swift amplified immune response observed following re-exposure to an antigen (Crotty, Aubert et al. 2004; Pollard, Perrett et al. 2009). Enumerating the numbers of antigen-specific memory B cells may be used as a surrogate of immune memory, as immune response following vaccination is evident even when antigen-specific antibodies are no longer evident (Nanan, Heinrich et al. 2001). Data presented in chapter 6 showed that DT-specific memory B cells in HIV positive vaccinees were lower than in HIV negative vaccinees. The impaired DT-specific memory B cell responses cannot be explained simply by the differences in the levels of memory B cells between the two groups because there were no significant differences between HIV-infected and uninfected individuals was seen. Impaired DT-specific memory B cell responses therefore may be a reflection of the fact that: 1) ART has not fully reversed intrinsic defects in the B cell response (Moir, Ogwaro et al. 2003), and/or 2) ART has not fully restored the ability of CD4 T cell to provide help to B cells leading to less optimal antibody responses. Poor and short-lived vaccine-induced memory B cell responses observed in HIV-infected person on ART indicates the need for booster doses of vaccines in HIV-infected persons to maintain a pool of antibody secreting cells necessary for sustained protection.

7.1.4 Impact of HIV infection, ART and PCV-7 vaccination on *S. pneumoniae* nasopharyngeal colonisation

HIV infection with or without progression to AIDS, significantly increases the risk of invasive disease due to the pneumococcus (Gilks CF, 1996, Dworkin MS, 2001). Nasopharyngeal colonization with *Streptococcus pneumoniae* precedes invasive pneumococcal disease (Bogaert, De Groot et al. 2004)). There has been conflicting data as to whether HIV infection increases the risk of pneumococcal nasopharyngeal colonisation. Recent literature however suggest that HIV infection increases the prevalence of colonisation in African adults (Gill, Mwanakasale et al. 2008) and children (Madhi, Adrian et al. 2007).

Data presented in this thesis show that HIV infection increases the frequency of pneumococcal nasopharyngeal colonization in HIV-infected Malawian adults with advanced disease but not in asymptomatic HIV-infected individuals (Table 7.1). 6 months ART or PCV7 vaccination of HIV-infected persons well established on ART did not reduce the nasopharyngeal carriage rates in HIV-infected adults (Table 7.1).

Table 7.1| Pneumococcal nasopharyngeal carriage rates in Malawian adults during HIV infection, ART and following PCV-7 vaccination.

	HIV-ve	HIV+ve (asymptomatic)	HIV+ve (Pre-ART)	HIV+ve (6 mths ART)	HIV+ve (6mths Post-Vac & >1yr ART)
N	31	50	48	42	27
Carriage (%)	5/31(16)	7/50(14)	13/48(27)	17/42(40)	9/27(33)

Pre-ART - individuals about to start ART; 6 mths post-vaccination – PCV7 vaccination

The observation that HIV infection increases the prevalence of colonisation may partly explain the increased rates of invasive pneumococcal disease in patients with HIV/AIDS. It may also be a reflection of compromised epithelial layer in the upper respiratory tract, innate and adaptive immunity.

7.2 Study limitations

Streptococcus pneumoniae is primarily a mucosal commensal colonising the nasopharynx. Natural carriage of pneumococcus and related bacteria in the nasopharynx leads to the development of 'protective' T cell immunity both at the mucosal surface and in the circulation (Zhang, Bagrade et al. 2007; Mureithi, Finn et al. 2009; Richards, Ferreira et al. 2010). This study mainly looked at the influence of HIV on pneumococcal responses in the periphery and as such they may not entirely reflect the effect of HIV on mucosal upper respiratory tract immunity. Recently, we investigated immune responses generated at mucosal pulmonary site which is an immune effector site (as compared to upper respiratory tract which is an inductive site).

We observed that the percentage frequencies of *S. pneumoniae*-antigen specific CD4+ T cells were higher in BAL than peripheral blood in HIV-uninfected (immunity is compartmentalized) (Jambo, Sepako et al. 2011). The data clearly indicated that solely investigating peripheral immune responses does not necessarily reflect responses generated at mucosal sites. Future studies would benefit if both and local mucosal responses to pneumococcal proteins were assessed. At the time the study (described and discussed in chapter 4) was undertaken, upper respiratory mucosal T cell sampling at Queen Elizabeth Central Hospital, Blantyre was not feasible. Such studies are now underway.

In this thesis, concentrated culture supernatants (CCS) derived from a standard strain D39 wild-type and isogenic mutant strains lacking pneumolysin (Ply-) were to measure antigen-specific CD4+ T cell responses. The limitation with using concentrated culture supernatant is that the native pneumococcal proteins in the CCS may be complex compared to the purified recombinant proteins, which may affect their uptake, processing and presentation. Additionally, concentrated culture supernatants may contain proteins also expressed by other similar bacteria. Nevertheless, using concentrated culture supernatant guaranteed that potentially the totality of pneumococcal-specific CD4+ T cell response was captured. The use of pneumoCCS in our *in vitro* stimulation most probably mimicked the *in-vivo* process of pneumococcal antigen presentation. In addition, some purified pneumococcal proteins such as pneumolysin are highly toxic to T cells in their native form.

For the study done in Chapter 6, the HIV negative study group (just like the HIV study participants) was drawn from individuals who have had a previous episode of invasive pneumococcal disease. Since pneumococcus rarely causes diseases in adults, our HIV negative group consisted of individuals with potentially compromised immune systems (subtle not gross defects) and therefore they were not a true representative of an HIV negative control group.

In some studies described in this thesis, especially where both T and B cell assays were done, it not always possible to do all the assays because of insufficient PBMCs. This was particularly true in Chapter 6. In such cases, assays had to be prioritised. Thus in some cases (for example cytokine responses to diphtheria toxoid), the sample size was small. However, the data collected could form the basis for future studies.

7.3 Future Studies

The data presented in this thesis raise a number of important mechanistic and clinical questions warranting further investigation. If addressed they would lead to a better understanding of the effects of HIV infection on pneumococcal immunity and reconstitution following antiretroviral therapy and could help in clinical care of HIV-infected persons.

7.3.1 Effects of HIV infection on pneumococcal immunity at the upper respiratory tract mucosal site

Studies show that HIV affects mucosal immunity and much earlier than systemic immunity (Guadalupe, Reay et al. 2003; Brenchley, Schacker et al. 2004; Mehandru, Poles et al. 2004). So far studies have focussed mainly on systemic immunity and immunity at the gut and pulmonary mucosal sites. Little is known about the effects of HIV on immunity at the upper respiratory tract. Taking into account that the pneumococcus is primarily a mucosal commensal colonising the nasopharynx and mucosal surface plays a significant role in the development of naturally acquired immunity to *S. pneumoniae*, understanding the effects of HIV on the upper respiratory tract and mucosal responses to pneumococcus may help explain why HIV –infected individuals have an increased risk of IPD.

7.3.2 Pneumococcal immunity following concurrent administration of ART and vaccine

WHO recommends that ART should be initiated before CD4 T cell count falls below 350 cells/μl regardless of symptoms (WHO 2009). As stated earlier, it is doubtful if this recommendation will be implemented in resource poor setting (at least in the short term), as for a variety of social and infrastructural reasons (Van Damme 2006; USAID 2010), people continue to access HIV testing and ART care at a late stage in their disease. Therefore, many HIV-infected individuals will still start ART with CD4 count <350 cells/μl. Data presented in chapter 5 demonstrated that there is some regeneration of pneumococcal immunity in HIV-infected persons on ART, the immunity is not as robust as seen in HIV negative persons suggesting that individuals on ART (especially in early stages of therapy) may need additional protection probably in the form of vaccination.

There are no WHO recommendations with regard to the use of conjugate vaccines in HIV positive adults yet and because of suboptimal activity of the 23-valent pneumococcal polysaccharide vaccine in HIV-infected adults, routine PPV23 vaccination of HIV-infected adults not recommended for use in resource poor countries (WHO 2008d). However, WHO recommends the use of other measures that directly or indirectly may help prevent pneumococcal disease such as trimethoprim-sulfamethoxazole chemoprophylaxis (WHO 2008d). Nonetheless, a recent study done in Malawi, the 7-valent pneumococcal conjugate vaccine protected HIV-infected adults from recurrent pneumococcal infection caused by vaccine serotypes or serotype 6A supporting a possible role for the 7-valent or similarly constructed vaccine among HIV-positive adults. Further studies should aim to understand whether there are any benefits in vaccinating individuals (perhaps with pneumococcal conjugate vaccines) starting antiretroviral therapy especially those with CD4 count <350 cells/µl. The studies should also help define the optimal time, frequency and clinical effectiveness of additional doses.

7.3.3 Longevity of regenerated immune responses against pneumococcal protein antigens

Data presented in chapter 5 indicated that there is some restoration of pneumococcal CD4+ T cell immunity in HIV-infected persons by 6 months ART. Further data will be generated on this cohort by the MLW study team at 12 months ART. Studies have shown that there is either poor restoration of antigen-specific immune responses in individuals who are severely immunodeficient prior to treatment (Lange, Valdez et al. 2002; Sieg, Mitchem et al. 2002; Lederman, Williams et al. 2003; French, Keane et al. 2007) or the restoration is short-lived in this group (Keane, Price et al. 2004). An important and unanswered question is whether the reconstituted pneumococcal immunity observed in chapter 5 is preserved long-term especially in a setting like Malawi where pneumococcal carriage rates are very high. Future studies should therefore aim to assess the robustness and protective efficacy of immunity against pneumococcal protein antigens following long-term ART. A proposed study design would include following individuals on ART beyond 12 months and collect samples during their routine clinical visits and assess pneumococcal immunity.

7.3.4 Effects of HIV and ART on nasopharyngeal flora and pneumococcal Serotypes

Our data show that individuals commencing on ART have higher pneumococcal carriage rates compared to HIV-uninfected and these rates remained relatively unchanged in the first 6 months of ART. Questions remain as to whether HIIV infection alters: (i) the nasopharyngeal flora; (ii) the serotypes of the colonising *S. pneumoniae* (iii) the number of colonising pneumococcus and if so does ART in the long-term restore the nasopharyngeal flora and colonising pneumococcal serotypes. Until recently, it was almost possible to answer these questions. Serotyping methodologies were limited in the coverage of serotypes or detection of multiple serotype carriage. A microarray-based molecular serotyping method has now been developed (Hinds and Turner 2010). The method provided the ability to detect and identify multiple serotype carriage, co-colonization by other pathogens and also determine the relative abundance of each serotype present. The presence of multiple serotypes is detected in DNA extracted from a plate sweep of bacterial colonies grown from a nasopharyngeal swab. In the study described in chapter 5, nasopharyngeal swabs were collected before initiation of ART and at month 3, 6 and will be collected at 12 months after initiation of therapy. Therefore, these swabs can be used to answer the questions raised above.

7.4 Concluding Remarks

The findings of this thesis show that HIV compromises systemic pneumococcal immunity at an early stage in disease progression. This immunity is regenerated to some extent following initiation of ART. It remains to be seen however whether the reconstituted immunity is long-term and protective. *S. pneumoniae* is a highly adapted commensal and so the challenge particularly in an immunocompromised population is to generate robust long lasting protective immunity. In fact, data presented in this thesis suggest that immune responses that may be elicited by vaccines based on pneumococcal proteins may be impaired and possibly short-lived in HIV-infected even those on ART.

Since *S. pneumoniae* is a mucosal commensal and mucosal surface plays a significant role in the development of naturally acquired immunity to the pneumococcus, solely investigating the influence of HIV on pneumococcal immunity in the periphery may not fully explain why HIV-infected individuals are at an increased risk of IPD. There is a need for research on pneumococcal immunity at the upper respiratory tract mucosal sites. Nonetheless, the findings of this thesis have important implications for clinical care of HIV-infected persons on ART and future studies on pneumococcal immunity in the context of HIV infection and therapy.

REFERENCES

Aandahl, E. M., J. Michaelsson, et al. (2004). "Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens." J Virol **78**(5): 2454-9.

Abbas, A. K. and A. H. Lichtman (2007). Cellular and Molecular Immunology. *updated edition*, Elsevier Inc.

Abdullahi, O., J. Nyiro, et al. (2008). "The descriptive epidemiology of Streptococcus pneumoniae and Haemophilus influenzae nasopharyngeal carriage in children and adults in Kilifi district, Kenya." Pediatr Infect Dis J **27**(1): 59-64.

Abeyta, M., G. G. Hardy, et al. (2003). "Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of Streptococcus pneumoniae." Infect Immun **71**(1): 218-25.

Adam, D. (2009). "Issues in pneumococcal disease and pneumococcal conjugate vaccines: highlights of the 27th meeting of ESPID, Brussels, Belgium, June 9-13, 2009." Vaccine **27**(50): 7133-7.

Adegbola, R. A., E. K. Mulholland, et al. (1998). "Vaccination with a Haemophilus influenzae type b conjugate vaccine reduces oropharyngeal carriage of H. influenzae type b among Gambian children." J Infect Dis **177**(6): 1758-61.

Adegbola, R. A., S. O. Usen, et al. (1999). "Haemophilus influenzae type b meningitis in The Gambia after introduction of a conjugate vaccine." Lancet **354**(9184): 1091-2.

Agematsu, K., S. Hokibara, et al. (2000). "CD27: a memory B-cell marker." Immunol Today **21**(5): 204-6.

Altman, J. D., P. A. Moss, et al. (1996). "Phenotypic analysis of antigen-specific T lymphocytes." Science **274**(5284): 94-6.

Amyes, E., A. J. McMichael, et al. (2005). "Human CD4+ T cells are predominantly distributed among six phenotypically and functionally distinct subsets." J Immunol **175**(9): 5765-73.

Anthony, K. B., C. Yoder, et al. (2003). "Incomplete CD4 T cell recovery in HIV-1 infection after 12 months of highly active antiretroviral therapy is associated with ongoing increased CD4 T cell activation and turnover." J Acquir Immune Defic Syndr **33**(2): 125-33.

Appay, V. and D. Sauce (2008). "Immune activation and inflammation in HIV-1 infection: causes and consequences." J Pathol **214**(2): 231-41.

Areas, A. P., M. L. Oliveira, et al. (2004). "Expression and characterization of cholera toxin B-pneumococcal surface adhesin A fusion protein in Escherichia coli: ability of CTB-PsaA to induce humoral immune response in mice." Biochem Biophys Res Commun **321**(1): 192-6.

Arevalo, M. T., Q. Xu, et al. (2009). "Mucosal vaccination with a multicomponent adenovirus-vectored vaccine protects against Streptococcus pneumoniae infection in the lung." FEMS Immunol Med Microbiol **55**(3): 346-51.

Audouy, S. A., M. L. van Roosmalen, et al. (2006). "Lactococcus lactis GEM particles displaying pneumococcal antigens induce local and systemic immune responses following intranasal immunization." Vaccine **24**(26): 5434-41.

Austrian, R. (1999). "The pneumococcus at the millennium: not down, not out." J Infect Dis **179 Suppl 2**: S338-41.

Balachandran, P., A. Brooks-Walter, et al. (2002). "Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of Streptococcus pneumoniae." Infect Immun **70**(5): 2526-34.

Baril, L., D. E. Briles, et al. (2004). "Characterization of antibodies to PspA and PsaA in adults over 50 years of age with invasive pneumococcal disease." Vaccine **23**(6): 789-93.

Baril, L., J. Dietemann, et al. (2006). "Pneumococcal surface protein A (PspA) is effective at eliciting T cell-mediated responses during invasive pneumococcal disease in adults." Clin Exp Immunol **145**(2): 277-86.

Basset, A., C. M. Thompson, et al. (2007). "Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins." Infect Immun **75**(11): 5460-4.

Bekker, V., H. Scherpbier, et al. (2006). "Persistent humoral immune defect in highly active antiretroviral therapy-treated children with HIV-1 infection: loss of specific antibodies against attenuated vaccine strains and natural viral infection." Pediatrics **118**(2): e315-22.

Belkaid, Y. (2007). "Regulatory T cells and infection: a dangerous necessity." Nat Rev Immunol **7**(11): 875-88.

Benito, J. M., M. Lopez, et al. (2005). "CD4+ T cell recovery beyond the first year of complete suppression of viral replication during highly active antiretroviral therapy is not influenced by CD8+ T cell activation." J Infect Dis **192**(12): 2142-6.

Benton, K. A., M. P. Everson, et al. (1995). "A pneumolysin-negative mutant of Streptococcus pneumoniae causes chronic bacteremia rather than acute sepsis in mice." Infect Immun **63**(2): 448-55.

Benveniste, O., A. Flahault, et al. (2005). "Mechanisms involved in the low-level regeneration of CD4+ cells in HIV-1-infected patients receiving highly active antiretroviral therapy who have prolonged undetectable plasma viral loads." J Infect Dis **191**(10): 1670-9.

Bergmann, S. and S. Hammerschmidt (2006). "Versatility of pneumococcal surface proteins." Microbiology **152**(Pt 2): 295-303.

Berkley, J. A., B. S. Lowe, et al. (2005). "Bacteremia among children admitted to a rural hospital in Kenya." N Engl J Med **352**(1): 39-47.

Bernasconi, N. L., E. Traggiai, et al. (2002). "Maintenance of serological memory by polyclonal activation of human memory B cells." Science **298**(5601): 2199-202.

Bernatoniene, J. and A. Finn (2005). "Advances in pneumococcal vaccines: advantages for infants and children." Drugs **65**(2): 229-55.

Bernatoniene, J., Q. Zhang, et al. (2008). "Induction of CC and CXC chemokines in human antigen-presenting dendritic cells by the pneumococcal proteins pneumolysin and CbpA, and the role played by toll-like receptor 4, NF-kappaB, and mitogen-activated protein kinases." J Infect Dis **198**(12): 1823-33.

Berry, A. M., R. A. Lock, et al. (1996). "Cloning and characterization of nanB, a second Streptococcus pneumoniae neuraminidase gene, and purification of the NanB enzyme from recombinant Escherichia coli." J Bacteriol **178**(16): 4854-60.

Berry, A. M., A. D. Ogunniyi, et al. (1999). "Comparative virulence of Streptococcus pneumoniae strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene." Infect Immun **67**(2): 981-5.

Berzins, S. P., A. P. Uldrich, et al. (2002). "Thymic regeneration: teaching an old immune system new tricks." Trends Mol Med **8**(10): 469-76.

Betts, M. R., J. M. Brenchley, et al. (2003). "Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation." J Immunol Methods **281**(1-2): 65-78.

Betts, M. R., M. C. Nason, et al. (2006). "HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells." Blood **107**(12): 4781-9.

Beutler, B. (2004). "Inferences, questions and possibilities in Toll-like receptor signalling." Nature **430**(6996): 257-63.

Beverly, B., S. M. Kang, et al. (1992). "Reversal of in vitro T cell clonal anergy by IL-2 stimulation." Int Immunol **4**(6): 661-71.

Bleul, C. C., L. Wu, et al. (1997). "The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes." Proc Natl Acad Sci U S A **94**(5): 1925-30.

Bogaert, D., R. De Groot, et al. (2004). "Streptococcus pneumoniae colonisation: the key to pneumococcal disease." Lancet Infect Dis **4**(3): 144-54.

Bogaert, D., M. N. Engelen, et al. (2001). "Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study." J Clin Microbiol **39**(9): 3316-20.

Bolton, D. L. and M. Roederer (2009). "Flow cytometry and the future of vaccine development." Expert Rev Vaccines **8**(6): 779-89.

Boulnois, G. J., J. C. Paton, et al. (1991). "Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of Streptococcus pneumoniae." Mol Microbiol **5**(11): 2611-6.

Bourgeois, C., Z. Hao, et al. (2008). "Ablation of thymic export causes accelerated decay of naive CD4 T cells in the periphery because of activation by environmental antigen." Proc Natl Acad Sci U S A **105**(25): 8691-6.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248-54.

Braun, J. S., J. E. Sublett, et al. (2002). "Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis." J Clin Invest **109**(1): 19-27.

Brenchley, J. M. and D. C. Douek (2008). "HIV infection and the gastrointestinal immune system." Mucosal Immunol **1**(1): 23-30.

Brenchley, J. M., N. J. Karandikar, et al. (2003). "Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells." Blood **101**(7): 2711-20.

Brenchley, J. M., T. W. Schacker, et al. (2004). "CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract." J Exp Med **200**(6): 749-59.

Briles, D. E., E. Ades, et al. (2000). "Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of Streptococcus pneumoniae." Infect Immun **68**(2): 796-800.

Briles, D. E., S. Hollingshead, et al. (2000). "The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection." Vaccine **18**(16): 1707-11.

Briles, D. E., R. C. Tart, et al. (1998). "Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA)." Clin Microbiol Rev **11**(4): 645-57.

Brombacher, F., R. A. Kastelein, et al. (2003). "Novel IL-12 family members shed light on the orchestration of Th1 responses." Trends Immunol **24**(4): 207-12.

Brown, J. S., S. M. Gilliland, et al. (2002). "Characterization of pit, a Streptococcus pneumoniae iron uptake ABC transporter." Infect Immun **70**(8): 4389-98.

Brown, J. S., T. Hussell, et al. (2002). "The classical pathway is the dominant complement pathway required for innate immunity to Streptococcus pneumoniae infection in mice." Proc Natl Acad Sci U S A **99**(26): 16969-74.

Bucy, R. P., R. D. Hockett, et al. (1999). "Initial increase in blood CD4(+) lymphocytes after HIV antiretroviral therapy reflects redistribution from lymphoid tissues." J Clin Invest **103**(10): 1391-8.

Burleigh, L., P. Y. Lozach, et al. (2006). "Infection of dendritic cells (DCs), not DC-SIGN-mediated internalization of human immunodeficiency virus, is required for long-term transfer of virus to T cells." J Virol **80**(6): 2949-57.

Burnaugh, A. M., L. J. Frantz, et al. (2008). "Growth of Streptococcus pneumoniae on human glycoconjugates is dependent upon the sequential activity of bacterial exoglycosidases." J Bacteriol **190**(1): 221-30.

Cagigi, A., A. Nilsson, et al. (2008). "B cell immunopathology during HIV-1 infection: lessons to learn for HIV-1 vaccine design." Vaccine **26**(24): 3016-25.

Campos, I. B., M. Darrieux, et al. (2008). "Nasal immunization of mice with Lactobacillus casei expressing the Pneumococcal Surface Protein A: induction of antibodies, complement deposition and partial protection against Streptococcus pneumoniae challenge." Microbes Infect **10**(5): 481-8.

Cao, W., B. D. Jamieson, et al. (2009). "Regulatory T cell expansion and immune activation during untreated HIV type 1 infection are associated with disease progression." AIDS Res Hum Retroviruses **25**(2): 183-91.

Card, C. M., P. J. McLaren, et al. (2009). "Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of CD4(+)CD25(+)FOXP3(+) regulatory T cells." J Infect Dis **199**(9): 1318-22.

Carsetti, R., M. M. Rosado, et al. (2004). "Peripheral development of B cells in mouse and man." Immunol Rev **197**: 179-91.

Cartwright, K. (2002). "Pneumococcal disease in western Europe: burden of disease, antibiotic resistance and management." Eur J Pediatr **161**(4): 188-95.

Caugant, D. A., E. A. Hoiby, et al. (1994). "Asymptomatic carriage of Neisseria meningitidis in a randomly sampled population." J Clin Microbiol **32**(2): 323-30.

CDC(MMWR) (2005). "Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease--United States, 1998-2003." MMWR Morb Mortal Wkly Rep **54**(36): 893-7.

CDC[MMWR] (1984). Recommendations of the Immunization Practices Advisory Committee (ACIP) Update: Pneumococcal Polysaccharide Vaccine Usage -- United States CDC. **33(20):** 273-6.

Centlivre, M., M. Sala, et al. (2007). "In HIV-1 pathogenesis the die is cast during primary infection." AIDS **21**(1): 1-11.

Chattopadhyay, P. K., J. Yu, et al. (2005). "A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles." Nat Med **11**(10): 1113-7.

Chattopadhyay, P. K., J. Yu, et al. (2006). "Live-cell assay to detect antigen-specific CD4+ T-cell responses by CD154 expression." Nat Protoc **1**(1): 1-6.

Chen, W., W. Jin, et al. (2003). "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3." J Exp Med **198**(12): 1875-86.

Cheung, Y. B., S. M. Zaman, et al. (2009). "Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian children who participated in a 9-valent pneumococcal conjugate vaccine trial and in their younger siblings." Pediatr Infect Dis J **28**(11): 990-5.

Chirmule, N., T. W. McCloskey, et al. (1995). "HIV gp120 inhibits T cell activation by interfering with expression of costimulatory molecules CD40 ligand and CD80 (B71)." J Immunol **155**(2): 917-24.

Chong, Y., H. Ikematsu, et al. (2004). "Selective CD27+ (memory) B cell reduction and characteristic B cell alteration in drug-naive and HAART-treated HIV type 1-infected patients." AIDS Res Hum Retroviruses **20**(2): 219-26.

Chougnet, C. (2003). "Role of CD40 ligand dysregulation in HIV-associated dysfunction of antigen-presenting cells." J Leukoc Biol **74**(5): 702-9.

Chougnet, C. and S. Gessani (2006). "Role of gp120 in dendritic cell dysfunction in HIV infection." J Leukoc Biol **80**(5): 994-1000.

Cobb, B. A. and D. L. Kasper (2005). "Zwitterionic capsular polysaccharides: the new MHCII-dependent antigens." Cell Microbiol **7**(10): 1398-403.

Cockeran, R., R. Anderson, et al. (2002). "The role of pneumolysin in the pathogenesis of Streptococcus pneumoniae infection." Curr Opin Infect Dis **15**(3): 235-9.

Colino, J. and C. M. Snapper (2003). "Two distinct mechanisms for induction of dendritic cell apoptosis in response to intact Streptococcus pneumoniae." J Immunol **171**(5): 2354-65.

Crotty, S., R. D. Aubert, et al. (2004). "Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system." J Immunol Methods **286**(1-2): 111-22.

Curtis, M. M. and S. S. Way (2009). "Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens." Immunology **126**(2): 177-85.

Cutts, F. T., S. M. Zaman, et al. (2005). "Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial." Lancet **365**(9465): 1139-46.

D'Orsogna, L. J., R. G. Krueger, et al. (2007). "Circulating memory B-cell subpopulations are affected differently by HIV infection and antiretroviral therapy." AIDS **21**(13): 1747-52.

D'Souza, M., A. P. Fontenot, et al. (2007). "Programmed death 1 expression on HIV-specific CD4+ T cells is driven by viral replication and associated with T cell dysfunction." J Immunol **179**(3): 1979-87.

Dagan, R. (2009). "Impact of pneumococcal conjugate vaccine on infections caused by antibiotic-resistant Streptococcus pneumoniae." Clin Microbiol Infect **15 Suppl 3**: 16-20.

Dagan, R., N. Givon-Lavi, et al. (2005). "Serum serotype-specific pneumococcal anticapsular immunoglobulin g concentrations after immunization with a 9-valent conjugate pneumococcal vaccine correlate with nasopharyngeal acquisition of pneumococcus." J Infect Dis **192**(3): 367-76.

Day, C. L., D. E. Kaufmann, et al. (2006). "PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression." Nature **443**(7109): 350-4.

de Jager, W. and G. T. Rijkers (2006). "Solid-phase and bead-based cytokine immunoassay: a comparison." Methods **38**(4): 294-303.

De Milito, A. (2004). "B lymphocyte dysfunctions in HIV infection." Curr HIV Res **2**(1): 11-21.

De Milito, A., C. Morch, et al. (2001). "Loss of memory (CD27) B lymphocytes in HIV-1 infection." AIDS **15**(8): 957-64.

De Milito, A., A. Nilsson, et al. (2004). "Mechanisms of hypergammaglobulinemia and impaired antigen-specific humoral immunity in HIV-1 infection." Blood **103**(6): 2180-6.

De Rosa, S. C., F. X. Lu, et al. (2004). "Vaccination in humans generates broad T cell cytokine responses." J Immunol **173**(9): 5372-80.

Delobel, P., M. T. Nugeyre, et al. (2006). "Naive T-cell depletion related to infection by X4 human immunodeficiency virus type 1 in poor immunological responders to highly active antiretroviral therapy." J Virol **80**(20): 10229-36.

Deng, H., R. Liu, et al. (1996). "Identification of a major co-receptor for primary isolates of HIV-1." Nature **381**(6584): 661-6.

Desai, S. and A. Landay (2010). "Early immune senescence in HIV disease." Curr HIV/AIDS Rep **7**(1): 4-10.

Dessing, M. C., S. Florquin, et al. (2008). "Toll-like receptor 2 contributes to antibacterial defence against pneumolysin-deficient pneumococci." Cell Microbiol **10**(1): 237-46.

Divekar, A. A., D. M. Zaiss, et al. (2006). "Protein vaccines induce uncommitted IL-2-secreting human and mouse CD4 T cells, whereas infections induce more IFN-gamma-secreting cells." J Immunol **176**(3): 1465-73.

Dockrell, D. H., M. Lee, et al. (2001). "Immune-mediated phagocytosis and killing of Streptococcus pneumoniae are associated with direct and bystander macrophage apoptosis." J Infect Dis **184**(6): 713-22.

Dockrell, D. H., H. M. Marriott, et al. (2003). "Alveolar macrophage apoptosis contributes to pneumococcal clearance in a resolving model of pulmonary infection." J Immunol **171**(10): 5380-8.

Donaghy, H., A. Pozniak, et al. (2001). "Loss of blood CD11c(+) myeloid and CD11c(-) plasmacytoid dendritic cells in patients with HIV-1 infection correlates with HIV-1 RNA virus load." Blood **98**(8): 2574-6.

Douek, D. (2007). "HIV disease progression: immune activation, microbes, and a leaky gut." Top HIV Med **15**(4): 114-7.

Douek, D. C., J. M. Brenchley, et al. (2002). "HIV preferentially infects HIV-specific CD4+ T cells." Nature **417**(6884): 95-8.

Douek, D. C., R. D. McFarland, et al. (1998). "Changes in thymic function with age and during the treatment of HIV infection." Nature **396**(6712): 690-5.

Dunham, R. M., B. Cervasi, et al. (2008). "CD127 and CD25 expression defines CD4+ T cell subsets that are differentially depleted during HIV infection." J Immunol **180**(8): 5582-92.

Dworkin, M. S., J. W. Ward, et al. (2001). "Pneumococcal disease among human immunodeficiency virus-infected persons: incidence, risk factors, and impact of vaccination." Clin Infect Dis **32**(5): 794-800.

Elshal, M. F. and J. P. McCoy (2006). "Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA." Methods **38**(4): 317-23.

Eskola, J., T. Kilpi, et al. (2001). "Efficacy of a pneumococcal conjugate vaccine against acute otitis media." N Engl J Med **344**(6): 403-9.

Evans, T. G., W. Bonnez, et al. (1998). "Highly active antiretroviral therapy results in a decrease in CD8+ T cell activation and preferential reconstitution of the peripheral CD4+ T cell population with memory rather than naive cells." Antiviral Res **39**(3): 163-73.

Fedson, D. S. and J. A. Scott (1999). "The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known." Vaccine **17 Suppl 1**: S11-8.

Feikin, D. R., M. Davis, et al. (2003). "Antibiotic resistance and serotype distribution of Streptococcus pneumoniae colonizing rural Malawian children." Pediatr Infect Dis J **22**(6): 564-7.

Feng, Y., C. C. Broder, et al. (1996). "HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor." Science **272**(5263): 872-7.

Ferguson, A. R., M. E. Youd, et al. (2004). "Marginal zone B cells transport and deposit IgM-containing immune complexes onto follicular dendritic cells." Int Immunol **16**(10): 1411-22.

Ferreira, D. M., M. Darrieux, et al. (2009). "Characterization of protective mucosal and systemic immune responses elicited by pneumococcal surface protein PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice." Clin Vaccine Immunol **16**(5): 636-45.

Flannery, B., S. Schrag, et al. (2004). "Impact of childhood vaccination on racial disparities in invasive Streptococcus pneumoniae infections." JAMA **291**(18): 2197-203.

Foy, H. M., M. K. Cooney, et al. (1973). "Incidence and etiology of pneumonia, croup and bronchiolitis in preschool children belonging to a prepaid medical care group over a four-year period." Am J Epidemiol **97**(2): 80-92.

French, M., N. Keane, et al. (2007). "Susceptibility to opportunistic infections in HIV-infected patients with increased CD4 T-cell counts on antiretroviral therapy may be predicted by markers of dysfunctional effector memory CD4 T cells and B cells." HIV Med **8**(3): 148-55.

French, N. (2003). "Use of pneumococcal polysaccharide vaccines: no simple answers." J Infect **46**(2): 78-86.

French, N., S. B. Gordon, et al. (2010). "A trial of a 7-valent pneumococcal conjugate vaccine in HIV-infected adults." N Engl J Med **362**(9): 812-22.

French, N., J. Nakiyingi, et al. (2000). "23-valent pneumococcal polysaccharide vaccine in HIV-1-infected Ugandan adults: double-blind, randomised and placebo controlled trial." Lancet **355**(9221): 2106-11.

Frentsch, M., O. Arbach, et al. (2005). "Direct access to CD4+ T cells specific for defined antigens according to CD154 expression." Nat Med **11**(10): 1118-24.

Furco, A., M. Carmagnat, et al. (2008). "Restoration of Toxoplasma gondii-specific immune responses in patients with AIDS starting HAART." AIDS **22**(16): 2087-96.

Gaardbo, J. C., S. D. Nielsen, et al. (2008). "Regulatory T cells in human immunodeficiency virus-infected patients are elevated and independent of immunological and virological status, as well as initiation of highly active anti-retroviral therapy." Clin Exp Immunol **154**(1): 80-6.

Gandhi, R. T., J. Spritzler, et al. (2006). "Effect of baseline- and treatment-related factors on immunologic recovery after initiation of antiretroviral therapy in HIV-1-positive subjects: results from ACTG 384." J Acquir Immune Defic Syndr **42**(4): 426-34.

Gazzola, L., C. Tincati, et al. (2009). "The absence of CD4+ T cell count recovery despite receipt of virologically suppressive highly active antiretroviral therapy: clinical risk, immunological gaps, and therapeutic options." Clin Infect Dis **48**(3): 328-37.

Ge, Q., H. Hu, et al. (2002). "Different contributions of thymopoiesis and homeostasis-driven proliferation to the reconstitution of naive and memory T cell compartments." Proc Natl Acad Sci U S A **99**(5): 2989-94.

Geginat, J., F. Sallusto, et al. (2001). "Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells." J Exp Med **194**(12): 1711-9.

Geldmacher, C., N. Ngwenyama, et al. (2010). "Preferential infection and depletion of Mycobacterium tuberculosis-specific CD4 T cells after HIV-1 infection." J Exp Med **207**(13): 2869-81.

Geng, E. H. and S. G. Deeks (2009). "CD4+ T cell recovery with antiretroviral therapy: more than the sum of the parts." Clin Infect Dis **48**(3): 362-4.

Ghaffar, F., T. Barton, et al. (2004). "Effect of the 7-valent pneumococcal conjugate vaccine on nasopharyngeal colonization by Streptococcus pneumoniae in the first 2 years of life." Clin Infect Dis **39**(7): 930-8.

Giefing, C., A. L. Meinke, et al. (2008). "Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies." J Exp Med **205**(1): 117-31.

Gill, C. J., V. Mwanakasale, et al. (2008). "Impact of human immunodeficiency virus infection on Streptococcus pneumoniae colonization and seroepidemiology among Zambian women." J Infect Dis **197**(7): 1000-5.

Goicoechea, M., D. M. Smith, et al. (2006). "Determinants of CD4+ T cell recovery during suppressive antiretroviral therapy: association of immune activation, T cell maturation markers, and cellular HIV-1 DNA." J Infect Dis **194**(1): 29-37.

Goldblatt, D., M. Hussain, et al. (2005). "Antibody responses to nasopharyngeal carriage of Streptococcus pneumoniae in adults: a longitudinal household study." J Infect Dis **192**(3): 387-93.

Goldsby, R. A., T. K. Kindt, et al. (2003). Immunology. New York, W.H. Freeman and Company.

Gordon, S. B., M. Chaponda, et al. (2002). "Pneumococcal disease in HIV-infected Malawian adults: acute mortality and long-term survival." AIDS **16**(10): 1409-17.

Gordon, S. B., S. Kanyanda, et al. (2003). "Poor potential coverage for 7-valent pneumococcal conjugate vaccine, Malawi." Emerg Infect Dis **9**(6): 747-9.

Gorry, P. R. and P. Ancuta (2011). "Coreceptors and HIV-1 pathogenesis." Curr HIV/AIDS Rep **8**(1): 45-53.

Granat, S. M., Z. Mia, et al. (2007). "Longitudinal study on pneumococcal carriage during the first year of life in Bangladesh." Pediatr Infect Dis J **26**(4): 319-24.

Granat, S. M., J. Ollgren, et al. (2009). "Epidemiological evidence for serotype-independent acquired immunity to pneumococcal carriage." J Infect Dis **200**(1): 99-106.

Grau, I., R. Pallares, et al. (2005). "Epidemiologic changes in bacteremic pneumococcal disease in patients with human immunodeficiency virus in the era of highly active antiretroviral therapy." Arch Intern Med **165**(13): 1533-40.

Greenwood, B. (1999). "The epidemiology of pneumococcal infection in children in the developing world." Philos Trans R Soc Lond B Biol Sci **354**(1384): 777-85.

Grossman, Z., M. Meier-Schellersheim, et al. (2002). "CD4+ T-cell depletion in HIV infection: are we closer to understanding the cause?" Nat Med **8**(4): 319-23.

Guadalupe, M., E. Reay, et al. (2003). "Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy." J Virol **77**(21): 11708-17.

Guinamard, R., M. Okigaki, et al. (2000). "Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response." Nat Immunol **1**(1): 31-6.

Gwaltney, J. M., Jr., M. A. Sande, et al. (1975). "Spread of Streptococcus pneumoniae in families. II. Relation of transfer of S. pneumoniae to incidence of colds and serum antibody." J Infect Dis **132**(1): 62-8.

Hansen, A. K. (2000). Handbook of laboratory Animal bacteriology. London, CRC Press.

Happel, K. I., P. J. Dubin, et al. (2005). "Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae." J Exp Med **202**(6): 761-9.

Harari, A., F. Vallelian, et al. (2004). "Phenotypic heterogeneity of antigen-specific CD4 T cells under different conditions of antigen persistence and antigen load." Eur J Immunol **34**(12): 3525-33.

Hardy, G. A., N. Imami, et al. (2003). "Reconstitution of CD4+ T cell responses in HIV-1 infected individuals initiating highly active antiretroviral therapy (HAART) is associated with renewed interleukin-2 production and responsiveness." Clin Exp Immunol **134**(1): 98-106.

Harrison, L. M., J. A. Morris, et al. (1999). "The nasopharyngeal bacterial flora in infancy: effects of age, gender, season, viral upper respiratory tract infection and sleeping position." FEMS Immunol Med Microbiol **25**(1-2): 19-28.

Hart, M., A. Steel, et al. (2007). "Loss of discrete memory B cell subsets is associated with impaired immunization responses in HIV-1 infection and may be a risk factor for invasive pneumococcal disease." J Immunol **178**(12): 8212-20.

Hazenberg, M. D., D. Hamann, et al. (2000). "T cell depletion in HIV-1 infection: how CD4+ T cells go out of stock." Nat Immunol **1**(4): 285-9.

Hazra, R. and C. Mackall (2005). "Thymic function in HIV infection." Curr HIV/AIDS Rep **2**(1): 24-8.

Heffernan, R. T., N. L. Barrett, et al. (2005). "Declining incidence of invasive Streptococcus pneumoniae infections among persons with AIDS in an era of highly active antiretroviral therapy, 1995-2000." J Infect Dis **191**(12): 2038-45.

Henneke, P., O. Takeuchi, et al. (2002). "Cellular activation, phagocytosis, and bactericidal activity against group B streptococcus involve parallel myeloid differentiation factor 88-dependent and independent signaling pathways." J Immunol **169**(7): 3970-7.

Heritage J, E. E. G., Killington R.A (1996). Introductory Microbiology Cambridge University Press.

Heyderman, R. S., V. Davenport, et al. (2006). "Mucosal immunity and optimizing protection with meningococcal serogroup B vaccines." Trends Microbiol **14**(3): 120-4.

Hickling, J. K. (1998). "Measuring human T-lymphocyte function." Expert Rev Mol Med **1998**: 1-20.

Hicks, L. A., L. H. Harrison, et al. (2007). "Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998-2004." J Infect Dis **196**(9): 1346-54.

Higgins, S. C., A. G. Jarnicki, et al. (2006). "TLR4 mediates vaccine-induced protective cellular immunity to Bordetella pertussis: role of IL-17-producing T cells." J Immunol **177**(11): 7980-9.

Hill, P. C., A. Akisanya, et al. (2006). "Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian villagers." Clin Infect Dis **43**(6): 673-9.

Hinds, J., Gould, K.A., Witney, A.A., Baldry, S.J., Lambertsen, L., Hanage, W.P., Antonio, M., and P. Turner, Newton R.H., Wernisch, L., Aanensen, D.M. and Bentley, S.D. (2010). "Investigation of serotype diversity, non-typeable isolates and nasopharygeal co-colonization by microarray-based

molecular serotyping of Streptococcus pneumoniae." Retrieved 9 March, 2011, from www.meningitis.org/assests/x/52303.

Hirst, R. A., A. Kadioglu, et al. (2004). "The role of pneumolysin in pneumococcal pneumonia and meningitis." Clin Exp Immunol **138**(2): 195-201.

Hogberg, L., P. Geli, et al. (2007). "Age- and serogroup-related differences in observed durations of nasopharyngeal carriage of penicillin-resistant pneumococci." J Clin Microbiol **45**(3): 948-52.

Horton, H., I. Frank, et al. (2006). "Preservation of T cell proliferation restricted by protective HLA alleles is critical for immune control of HIV-1 infection." J Immunol **177**(10): 7406-15.

Hostetter, M. K. (2004). Interactions of *Streptococcus pneumoniae* with the proteins of the complement pathway. The pneumococcus. E. I. Tuomanen, Mitchell, T.J., Morisson, D.A., Spratt, B.G. Washington DC, American Society for Microbiology: 2001-210.

Howden, R. (1976). "Use of anaerobic culture for the improved isolation of Streptococcus pneumoniae." J Clin Pathol **29**(1): 50-3.

HPA(UK) (2004). Optochin Test. National Standard Method BSOP TP, Health Protection Agency

Huang, S. S., V. L. Hinrichsen, et al. (2009). "Continued impact of pneumococcal conjugate vaccine on carriage in young children." Pediatrics **124**(1): e1-11.

Huang, W., L. Na, et al. (2004). "Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice." J Infect Dis **190**(3): 624-31.

Hunt, P. W., S. G. Deeks, et al. (2003). "Continued CD4 cell count increases in HIV-infected adults experiencing 4 years of viral suppression on antiretroviral therapy." AIDS **17**(13): 1907-15.

Hwang, Y., M. H. Nahm, et al. (2000). "Acquired, but not innate, immune responses to Streptococcus pneumoniae are compromised by neutralization of CD40L." Infect Immun **68**(2): 511-7.

Jain, A., T. P. Atkinson, et al. (1999). "Defects of T-cell effector function and post-thymic maturation in X-linked hyper-IgM syndrome." J Clin Invest **103**(8): 1151-8.

Jakobsen, H., E. Saeland, et al. (1999). "Intranasal immunization with pneumococcal polysaccharide conjugate vaccines protects mice against invasive pneumococcal infections." Infect Immun **67**(8): 4128-33.

Jambo, K. C., E. Sepako, et al. (2011). "Bronchoalveolar CD4+ T cell responses to respiratory antigens are impaired in HIV-infected adults." Thorax.

Jambo, K. C., E. Sepako, et al. (2010). "Potential role for mucosally active vaccines against pneumococcal pneumonia." Trends Microbiol **18**(2): 81-9.

Jarva, H., J. Hellwage, et al. (2004). "The group B streptococcal beta and pneumococcal Hic proteins are structurally related immune evasion molecules that bind the complement inhibitor factor H in an analogous fashion." J Immunol **172**(5): 3111-8.

Jedrzejas, M. J. (2001). "Pneumococcal virulence factors: structure and function." Microbiol Mol Biol Rev **65**(2): 187-207 ; first page, table of contents.

Jeurissen, A., A. D. Billiau, et al. (2006). "CD4+ T lymphocytes expressing CD40 ligand help the IgM antibody response to soluble pneumococcal polysaccharides via an intermediate cell type." J Immunol **176**(1): 529-36.

Jiang, W., M. M. Lederman, et al. (2009). "Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection." J Infect Dis **199**(8): 1177-85.

Johnson, S. E., J. K. Dykes, et al. (2002). "Inhibition of pneumococcal carriage in mice by subcutaneous immunization with peptides from the common surface protein pneumococcal surface adhesin a." J Infect Dis **185**(4): 489-96.

Jomaa, M., J. Yuste, et al. (2005). "Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of Streptococcus pneumoniae." Infect Immun **73**(10): 6852-9.

Jones, N., R. Huebner, et al. (1998). "The impact of HIV on Streptococcus pneumoniae bacteraemia in a South African population." Aids **12**(16): 2177-84.

Jounblat, R., A. Kadioglu, et al. (2003). "Pneumococcal behavior and host responses during bronchopneumonia are affected differently by the cytolytic and complement-activating activities of pneumolysin." Infect Immun **71**(4): 1813-9.

Juven, T., J. Mertsola, et al. (2000). "Etiology of community-acquired pneumonia in 254 hospitalized children." Pediatr Infect Dis J **19**(4): 293-8.

Kadioglu, A., J. N. Weiser, et al. (2008). "The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease." Nat Rev Microbiol **6**(4): 288-301.

Kang, H. Y., J. Srinivasan, et al. (2002). "Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated Salmonella enterica serovar typhimurium vaccine." Infect Immun **70**(4): 1739-49.

Kaplan, J. E., D. Hanson, et al. (2000). "Epidemiology of human immunodeficiency virus-associated opportunistic infections in the United States in the era of highly active antiretroviral therapy." Clin Infect Dis **30 Suppl 1**: S5-14.

Karlsson, A. C., J. N. Martin, et al. (2003). "Comparison of the ELISPOT and cytokine flow cytometry assays for the enumeration of antigen-specific T cells." J Immunol Methods **283**(1-2): 141-53.

Karlsson, I., B. Malleret, et al. (2011). "Suppressive activity of regulatory T cells correlates with high CD4+ T-cell counts and low T-cell activation during chronic SIV infection." AIDS **25**(5): 585-593.

Keane, N. M., P. Price, et al. (2004). "Restoration of CD4 T-cell responses to cytomegalovirus is short-lived in severely immunodeficient HIV-infected patients responding to highly active antiretroviral therapy." HIV Med **5**(6): 407-14.

Kedzierska, K., R. Azzam, et al. (2003). "Defective phagocytosis by human monocyte/macrophages following HIV-1 infection: underlying mechanisms and modulation by adjunctive cytokine therapy." J Clin Virol **26**(2): 247-63.

Kedzierska, K. and S. M. Crowe (2002). "The role of monocytes and macrophages in the pathogenesis of HIV-1 infection." Curr Med Chem **9**(21): 1893-903.

Keele, B. F., E. E. Giorgi, et al. (2008). "Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection." Proc Natl Acad Sci U S A **105**(21): 7552-7.

Kelley, C. F., C. M. Kitchen, et al. (2009). "Incomplete peripheral CD4+ cell count restoration in HIV-infected patients receiving long-term antiretroviral treatment." Clin Infect Dis **48**(6): 787-94.

Keynan, Y., C. M. Card, et al. (2008). "The role of regulatory T cells in chronic and acute viral infections." Clin Infect Dis **46**(7): 1046-52.

King, S. J., K. R. Hippe, et al. (2004). "Phase variable desialylation of host proteins that bind to Streptococcus pneumoniae in vivo and protect the airway." Mol Microbiol **54**(1): 159-71.

Kinter, A. L., M. Hennessey, et al. (2004). "CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status." J Exp Med **200**(3): 331-43.

Kinter, A. L., R. Horak, et al. (2007). "CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells in vitro." AIDS Res Hum Retroviruses **23**(3): 438-50.

Kitahata, M. M., S. J. Gange, et al. (2009). "Effect of early versus deferred antiretroviral therapy for HIV on survival." N Engl J Med **360**(18): 1815-26.

Klebanoff, C. A., L. Gattinoni, et al. (2005). "Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells." Proc Natl Acad Sci U S A **102**(27): 9571-6.

Klein, U., K. Rajewsky, et al. (1998). "Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells." J Exp Med **188**(9): 1679-89.

Klugman, K. P., and Makela, P Helena , and Siber, George R, Ed. (2008). Pneumococcal Vaccines: the Impact of Conjugate Vaccine. Immunogenicity in High-Risk and Immunocompromised children and adults. Washington DC, ASM Press.

Klugman, K. P., and Makela, P Helena , and Siber, George R Ed. (2008). Pneumococcal Vaccines: the Impact of Conjugate Vaccine Epidemiology,Diagnosis and Treatment of serious pneumococcal infections in Children(pp 95-116). . Washington DC, ASM Press.

Klugman, K. P., S. A. Madhi, et al. (2007). "HIV and pneumococcal disease." Curr Opin Infect Dis **20**(1): 11-5.

Klugman, K. P., S. A. Madhi, et al. (2003). "A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection." N Engl J Med **349**(14): 1341-8.

Knox, K. S., C. Vinton, et al. (2010). "Reconstitution of CD4 T cells in bronchoalveolar lavage fluid after initiation of highly active antiretroviral therapy." J Virol **84**(18): 9010-8.

Koguchi, Y., T. J. Thauland, et al. (2007). "Preformed CD40 ligand exists in secretory lysosomes in effector and memory CD4+ T cells and is quickly expressed on the cell surface in an antigen-specific manner." Blood **110**(7): 2520-7.

Krakauer, T. (1999). "Immune response to staphylococcal superantigens." Immunol Res **20**(2): 163-73.

Kroon, F. P., J. T. van Dissel, et al. (1995). "Antibody response to diphtheria, tetanus, and poliomyelitis vaccines in relation to the number of CD4+ T lymphocytes in adults infected with human immunodeficiency virus." Clin Infect Dis **21**(5): 1197-203.

Kruetzmann, S., M. M. Rosado, et al. (2003). "Human immunoglobulin M memory B cells controlling Streptococcus pneumoniae infections are generated in the spleen." J Exp Med **197**(7): 939-45.

Kyaw, M. H., R. Lynfield, et al. (2006). "Effect of introduction of the pneumococcal conjugate vaccine on drug-resistant Streptococcus pneumoniae." N Engl J Med **354**(14): 1455-63.

Lakshman, R. and A. Finn (2002). "Meningococcal serogroup C conjugate vaccine." Expert Opin Biol Ther **2**(1): 87-96.

Lakshman, R., I. Jones, et al. (2001). "Safety of a new conjugate meningococcal C vaccine in infants." Arch Dis Child **85**(5): 391-7.

Lamoreaux, L., M. Roederer, et al. (2006). "Intracellular cytokine optimization and standard operating procedure." Nat Protoc **1**(3): 1507-16.

Lange, C. G., H. Valdez, et al. (2002). "CD4+ T-lymphocyte nadir and the effect of highly active antiretroviral therapy on phenotypic and functional immune restoration in HIV-1 infection." Clin Immunol **102**(2): 154-61.

Lederman, H. M., P. L. Williams, et al. (2003). "Incomplete immune reconstitution after initiation of highly active antiretroviral therapy in human immunodeficiency virus-infected patients with severe CD4+ cell depletion." J Infect Dis **188**(12): 1794-803.

Lederman, H. M. and J. A. Winkelstein (1985). "X-linked agammaglobulinemia: an analysis of 96 patients." Medicine (Baltimore) **64**(3): 145-56.

Lederman, M. M. (2001). "Immune restoration and CD4+ T-cell function with antiretroviral therapies." AIDS **15 Suppl 2**: S11-5.

Lee, S., M. A. French, et al. (2004). "IL-23 and IFN-gamma deficiency in immunodeficient HIV patients who achieved a long-term increase in CD4 T-cell counts on highly active antiretroviral therapy." AIDS **18**(9): 1337-40.

Leino, T., K. Auranen, et al. (2001). "Pneumococcal carriage in children during their first two years: important role of family exposure." Pediatr Infect Dis J **20**(11): 1022-7.

Levine, O. S., K. L. O'Brien, et al. (2006). "Pneumococcal vaccination in developing countries." Lancet **367**(9526): 1880-2.

Levy, J., T. Espanol-Boren, et al. (1997). "Clinical spectrum of X-linked hyper-IgM syndrome." J Pediatr **131**(1 Pt 1): 47-54.

Lewis SM, B. B., Bates I, Ed. (2005). Dacie and Lewis Practical Haematology. Edinburgh, Churchhill Livingstone.

Lexau, C. A., R. Lynfield, et al. (2005). "Changing epidemiology of invasive pneumococcal disease among older adults in the era of pediatric pneumococcal conjugate vaccine." JAMA **294**(16): 2043-51.

Li, Y., S. Wang, et al. (2009). "Evaluation of new generation Salmonella enterica serovar Typhimurium vaccines with regulated delayed attenuation to induce immune responses against PspA." Proc Natl Acad Sci U S A **106**(2): 593-8.

Libamba, E., S. D. Makombe, et al. (2007). "Malawi's contribution to "3 by 5": achievements and challenges." Bull World Health Organ **85**(2): 156-60.

Lim, A., D. Tan, et al. (2007). "Proportions of circulating T cells with a regulatory cell phenotype increase with HIV-associated immune activation and remain high on antiretroviral therapy." AIDS **21**(12): 1525-34.

Lipsitch, M., C. G. Whitney, et al. (2005). "Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease?" PLoS Med **2**(1): e15.

Llewelyn, M. and J. Cohen (2002). "Superantigens: microbial agents that corrupt immunity." Lancet Infect Dis **2**(3): 156-62.

Lock, R. A., D. Hansman, et al. (1992). "Comparative efficacy of autolysin and pneumolysin as immunogens protecting mice against infection by Streptococcus pneumoniae." Microb Pathog **12**(2): 137-43.

Lopes-Carvalho, T., J. Foote, et al. (2005). "Marginal zone B cells in lymphocyte activation and regulation." Curr Opin Immunol **17**(3): 244-50.

Lu, Y. J., J. Gross, et al. (2008). "Interleukin-17A mediates acquired immunity to pneumococcal colonization." PLoS Pathog **4**(9): e1000159.

Lu, Y. J., P. Yadav, et al. (2010). "Options for inactivation, adjuvant, and route of topical administration of a killed, unencapsulated pneumococcal whole-cell vaccine." Clin Vaccine Immunol **17**(6): 1005-12.

Lynch, J. M., D. E. Briles, et al. (2003). "Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12." Infect Immun **71**(8): 4780-8.

Lynch, J. P., 3rd and G. G. Zhanel (2009). "Streptococcus pneumoniae: does antimicrobial resistance matter?" Semin Respir Crit Care Med **30**(2): 210-38.

Lynch, J. P., 3rd and G. G. Zhanel (2009). "Streptococcus pneumoniae: epidemiology, risk factors, and strategies for prevention." Semin Respir Crit Care Med **30**(2): 189-209.

Lynch, J. P., 3rd and G. G. Zhanel (2010). "Streptococcus pneumoniae: epidemiology and risk factors, evolution of antimicrobial resistance, and impact of vaccines." Curr Opin Pulm Med **16**(3): 217-25.

Lyons, A. B. (2000). "Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution." J Immunol Methods **243**(1-2): 147-54.

Ma, D. Y. and E. A. Clark (2009). "The role of CD40 and CD154/CD40L in dendritic cells." Semin Immunol **21**(5): 265-72.

Mac, L. C. and M. R. Kraus (1950). "Relation of virulence of pneumococcal strains for mice to the quantity of capsular polysaccharide formed in vitro." J Exp Med **92**(1): 1-9.

Macal, M., S. Sankaran, et al. (2008). "Effective CD4+ T-cell restoration in gut-associated lymphoid tissue of HIV-infected patients is associated with enhanced Th17 cells and polyfunctional HIV-specific T-cell responses." Mucosal Immunol **1**(6): 475-88.

Mackall, C. L. and R. E. Gress (1997). "Thymic aging and T-cell regeneration." Immunol Rev **160**: 91-102.

Madhi, S. A., P. Adrian, et al. (2007). "Long-term effect of pneumococcal conjugate vaccine on nasopharyngeal colonization by Streptococcus pneumoniae--and associated interactions with Staphylococcus aureus and Haemophilus influenzae colonization--in HIV-Infected and HIV-uninfected children." J Infect Dis **196**(11): 1662-6.

Madhi, S. A., L. Kuwanda, et al. (2005). "The impact of a 9-valent pneumococcal conjugate vaccine on the public health burden of pneumonia in HIV-infected and -uninfected children." Clin Infect Dis **40**(10): 1511-8.

Madhi, S. A. and S. I. Pelton (2008). Epidemiology,Diagnosis and Treatment of serious pneumococcal infections in Children. Pneumococcal Vaccines. K. P. Klugman, and Makela, P Helena , and Siber, George R. Washington DC, ASM Press: 95-116.

Madhi, S. A., K. Petersen, et al. (2000). "Impact of human immunodeficiency virus type 1 on the disease spectrum of Streptococcus pneumoniae in South African children." Pediatr Infect Dis J **19**(12): 1141-7.

Malaspina, A., S. Moir, et al. (2005). "Compromised B cell responses to influenza vaccination in HIV-infected individuals." J Infect Dis **191**(9): 1442-50.

Malley, R. (2010). "Antibody and cell-mediated immunity to Streptococcus pneumoniae: implications for vaccine development." J Mol Med **88**(2): 135-42.

Malley, R., P. Henneke, et al. (2003). "Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection." Proc Natl Acad Sci U S A **100**(4): 1966-71.

Malley, R., M. Lipsitch, et al. (2001). "Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci." Infect Immun **69**(8): 4870-3.

Malley, R., S. C. Morse, et al. (2004). "Multiserotype protection of mice against pneumococcal colonization of the nasopharynx and middle ear by killed nonencapsulated cells given intranasally with a nontoxic adjuvant." Infect Immun **72**(7): 4290-2.

Malley, R., A. Srivastava, et al. (2006). "Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide." Infect Immun **74**(4): 2187-95.

Malley, R., K. Trzcinski, et al. (2005). "CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization." Proc Natl Acad Sci U S A **102**(13): 4848-53.

Manco, S., F. Hernon, et al. (2006). "Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis." Infect Immun **74**(7): 4014-20.

Manfredi, R., A. Nanetti, et al. (2000). "Moraxella catarrhalis pneumonia during HIV disease." J Chemother **12**(5): 406-11.

Mannering, S. I., J. S. Morris, et al. (2003). "A sensitive method for detecting proliferation of rare autoantigen-specific human T cells." J Immunol Methods **283**(1-2): 173-83.

Marra, A., S. Lawson, et al. (2002). "In vivo characterization of the psa genes from Streptococcus pneumoniae in multiple models of infection." Microbiology **148**(Pt 5): 1483-91.

Marriott, H. M., T. J. Mitchell, et al. (2008). "Pneumolysin: a double-edged sword during the host-pathogen interaction." Curr Mol Med **8**(6): 497-509.

Marschner, S., T. Hunig, et al. (2002). "Ligation of human CD4 interferes with antigen-induced activation of primary T cells." Immunol Lett **82**(1-2): 131-9.

Martin, F. and J. F. Kearney (2002). "Marginal-zone B cells." Nat Rev Immunol **2**(5): 323-35.

Martin, F., A. M. Oliver, et al. (2001). "Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens." Immunity **14**(5): 617-29.

Martinez-Maza, O. and E. C. Breen (2002). "B-cell activation and lymphoma in patients with HIV." Curr Opin Oncol **14**(5): 528-32.

Mashishi, T. and C. M. Gray (2002). "The ELISPOT assay: an easily transferable method for measuring cellular responses and identifying T cell epitopes." Clin Chem Lab Med **40**(9): 903-10.

Masur, H., J. E. Kaplan, et al. (2002). "Guidelines for preventing opportunistic infections among HIV-infected persons--2002. Recommendations of the U.S. Public Health Service and the Infectious Diseases Society of America." Ann Intern Med **137**(5 Pt 2): 435-78.

Matthias, K. A., A. M. Roche, et al. (2008). "Neutrophil-toxin interactions promote antigen delivery and mucosal clearance of Streptococcus pneumoniae." J Immunol **180**(9): 6246-54.

Maurer, D., G. F. Fischer, et al. (1992). "IgM and IgG but not cytokine secretion is restricted to the CD27+ B lymphocyte subset." J Immunol **148**(12): 3700-5.

Maynard, C. L., L. E. Harrington, et al. (2007). "Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10." Nat Immunol **8**(9): 931-41.

Mbelle, N., R. E. Huebner, et al. (1999). "Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine." J Infect Dis **180**(4): 1171-6.

McCool, T. L., T. R. Cate, et al. (2002). "The immune response to pneumococcal proteins during experimental human carriage." J Exp Med **195**(3): 359-65.

McCool, T. L., T. R. Cate, et al. (2003). "Serum immunoglobulin G response to candidate vaccine antigens during experimental human pneumococcal colonization." Infect Immun **71**(10): 5724-32.

McCune, J. M. (2001). "The dynamics of CD4+ T-cell depletion in HIV disease." Nature **410**(6831): 974-9.

McDonald, D., L. Wu, et al. (2003). "Recruitment of HIV and its receptors to dendritic cell-T cell junctions." Science **300**(5623): 1295-7.

McEllistrem, M. C., A. B. Mendelsohn, et al. (2002). "Recurrent invasive pneumococcal disease in individuals with human immunodeficiency virus infection." J Infect Dis **185**(9): 1364-8.

McKinnon, L. R., M. Kimani, et al. (2010). "Effect of baseline HIV disease parameters on CD4+ T cell recovery after antiretroviral therapy initiation in Kenyan women." PLoS One **5**(7): e11434.

Mehandru, S., M. A. Poles, et al. (2004). "Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract." J Exp Med **200**(6): 761-70.

Merino, J., M. A. Martinez-Gonzalez, et al. (1998). "Progressive decrease of CD8high+ CD28+ CD57- cells with ageing." Clin Exp Immunol **112**(1): 48-51.

Meyaard, L., H. Schuitemaker, et al. (1993). "T-cell dysfunction in HIV infection: anergy due to defective antigen-presenting cell function?" Immunol Today **14**(4): 161-4.

Micheloud, D., J. Berenguer, et al. (2008). "Negative influence of age on CD4+ cell recovery after highly active antiretroviral therapy in naive HIV-1-infected patients with severe immunodeficiency." J Infect **56**(2): 130-6.

Miga, A., S. Masters, et al. (2000). "The role of CD40-CD154 interactions in the regulation of cell mediated immunity." Immunol Invest **29**(2): 111-4.

Millar, E. V., K. L. O'Brien, et al. (2006). "Effect of community-wide conjugate pneumococcal vaccine use in infancy on nasopharyngeal carriage through 3 years of age: a cross-sectional study in a high-risk population." Clin Infect Dis **43**(1): 8-15.

Millar, E. V., J. P. Watt, et al. (2008). "Indirect effect of 7-valent pneumococcal conjugate vaccine on pneumococcal colonization among unvaccinated household members." Clin Infect Dis **47**(8): 989-96.

Mitchell, T. J., P. W. Andrew, et al. (1991). "Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein." Mol Microbiol **5**(8): 1883-8.

Moens, L., G. Wuyts, et al. (2008). "The human polysaccharide- and protein-specific immune response to Streptococcus pneumoniae is dependent on CD4(+) T lymphocytes, CD14(+) monocytes, and the CD40-CD40 ligand interaction." J Allergy Clin Immunol **122**(6): 1231-3.

Mofenson, L. M., R. Yogev, et al. (1998). "Characteristics of acute pneumonia in human immunodeficiency virus-infected children and association with long term mortality risk. National Institute of Child Health and Human Development Intravenous Immunoglobulin Clinical Trial Study Group." Pediatr Infect Dis J **17**(10): 872-80.

Moir, S. and A. S. Fauci (2008). "Pathogenic mechanisms of B-lymphocyte dysfunction in HIV disease." J Allergy Clin Immunol **122**(1): 12-9; quiz 20-1.

Moir, S. and A. S. Fauci (2009). "B cells in HIV infection and disease." Nat Rev Immunol **9**(4): 235-45.

Moir, S., A. Malaspina, et al. (2008). "Normalization of B cell counts and subpopulations after antiretroviral therapy in chronic HIV disease." J Infect Dis **197**(4): 572-9.

Moir, S., K. M. Ogwaro, et al. (2003). "Perturbations in B cell responsiveness to CD4+ T cell help in HIV-infected individuals." Proc Natl Acad Sci U S A **100**(10): 6057-62.

Montes, M., D. E. Lewis, et al. (2006). "Foxp3+ regulatory T cells in antiretroviral-naive HIV patients." AIDS **20**(12): 1669-71.

Moore, M. R., R. E. Gertz, Jr., et al. (2008). "Population snapshot of emergent Streptococcus pneumoniae serotype 19A in the United States, 2005." J Infect Dis **197**(7): 1016-27.

Moore, R. D. and J. C. Keruly (2007). "CD4+ cell count 6 years after commencement of highly active antiretroviral therapy in persons with sustained virologic suppression." Clin Infect Dis **44**(3): 441-6.

Mulholland, E. K., A. Hoestermann, et al. (1994). "Haemophilus influenzae type b meningitis after three doses of vaccine." Lancet **343**(8900): 794.

Mulholland, E. K., O. O. Ogunlesi, et al. (1999). "Etiology of serious infections in young Gambian infants." Pediatr Infect Dis J **18**(10 Suppl): S35-41.

Munoz-Almagro, C., I. Jordan, et al. (2008). "Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine." Clin Infect Dis **46**(2): 174-82.

Mureithi, M. W., A. Finn, et al. (2009). "T cell memory response to pneumococcal protein antigens in an area of high pneumococcal carriage and disease." J Infect Dis **200**(5): 783-93.

Musher, D. M., J. E. Groover, et al. (1993). "Antibody to capsular polysaccharides of Streptococcus pneumoniae: prevalence, persistence, and response to revaccination." Clin Infect Dis **17**(1): 66-73.

Mustafa, A. S., F. Oftung, et al. (2000). "Multiple epitopes from the Mycobacterium tuberculosis ESAT-6 antigen are recognized by antigen-specific human T cell lines." Clin Infect Dis **30 Suppl 3**: S201-5.

Nagase, H., K. Agematsu, et al. (2001). "Mechanism of hypergammaglobulinemia by HIV infection: circulating memory B-cell reduction with plasmacytosis." Clin Immunol **100**(2): 250-9.

Nanan, R., D. Heinrich, et al. (2001). "Acute and long-term effects of booster immunisation on frequencies of antigen-specific memory B-lymphocytes." Vaccine **20**(3-4): 498-504.

Nandoskar, M., A. Ferrante, et al. (1986). "Inhibition of human monocyte respiratory burst, degranulation, phospholipid methylation and bactericidal activity by pneumolysin." Immunology **59**(4): 515-20.

Nayak, A. R., S. A. Tinge, et al. (1998). "A live recombinant avirulent oral Salmonella vaccine expressing pneumococcal surface protein A induces protective responses against Streptococcus pneumoniae." Infect Immun **66**(8): 3744-51.

Negredo, E., M. Massanella, et al. (2010). "Nadir CD4 T cell count as predictor and high CD4 T cell intrinsic apoptosis as final mechanism of poor CD4 T cell recovery in virologically suppressed HIV-infected patients: clinical implications." Clin Infect Dis **50**(9): 1300-8.

Nelson, A. L., A. M. Roche, et al. (2007). "Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance." Infect Immun **75**(1): 83-90.

O'Brien, K. L. and H. Nohynek (2003). "Report from a WHO Working Group: standard method for detecting upper respiratory carriage of Streptococcus pneumoniae." Pediatr Infect Dis J **22**(2): e1-11.

O'Shea, J. J., A. Ma, et al. (2002). "Cytokines and autoimmunity." Nat Rev Immunol **2**(1): 37-45.

O'Brien, K. L., R. Dagan, et al. (2008). Nasopharyngeal Carriage. Pneumococcal Vaccines: the Impact of Conjugate Vaccine. K. P. Klugman, and Makela, P Helena , and Siber, George R. Washington DC, ASM Press: 279 -300.

O'Brien KL, D. R., Makela PH (2008). Nasopharyngeal Carriage. Pneumococcal Vaccines: the Impact of Conjugate Vaccine. K. P. Klugman, and Makela, P Helena , and Siber, George R. Washington DC, ASM Press: 279 -300.

Obaro, S. and R. Adegbola (2002). "The pneumococcus: carriage, disease and conjugate vaccines." J Med Microbiol **51**(2): 98-104.

Obaro, S. K., R. A. Adegbola, et al. (2000). "Pneumococcal surface adhesin A antibody concentration in serum and nasopharyngeal carriage of Streptococcus pneumoniae in young African infants." Vaccine **19**(4-5): 411-2.

Oftung, F., E. Borka, et al. (1998). "Mycobacterium tuberculosis reactive T cell clones from naturally converted PPD-positive healthy subjects: recognition of the M. tuberculosis 16-kDa antigen." FEMS Immunol Med Microbiol **20**(4): 319-25.

Ogunniyi, A. D., K. S. Lemessurier, et al. (2007). "Contribution of Pneumolysin, PspA and PspC (CbpA) to Pathogenicity of Streptococcus pneumoniae D39 in a Mouse Model." Infect Immun.

Ogunniyi, A. D., K. S. LeMessurier, et al. (2007). "Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of Streptococcus pneumoniae D39 in a mouse model." Infect Immun **75**(4): 1843-51.

Oliveira, M. L., A. P. Areas, et al. (2006). "Induction of systemic and mucosal immune response and decrease in Streptococcus pneumoniae colonization by nasal inoculation of mice with recombinant lactic acid bacteria expressing pneumococcal surface antigen A." Microbes Infect **8**(4): 1016-24.

Orihuela, C. J., G. Gao, et al. (2004). "Tissue-specific contributions of pneumococcal virulence factors to pathogenesis." J Infect Dis **190**(9): 1661-9.

Oswald-Richter, K., S. M. Grill, et al. (2004). "HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells." PLoS Biol **2**(7): E198.

Pacanowski, J., S. Kahi, et al. (2001). "Reduced blood CD123+ (lymphoid) and CD11c+ (myeloid) dendritic cell numbers in primary HIV-1 infection." Blood **98**(10): 3016-21.

Pai, R., M. R. Moore, et al. (2005). "Postvaccine genetic structure of Streptococcus pneumoniae serotype 19A from children in the United States." J Infect Dis **192**(11): 1988-95.

Palaniappan, R., S. Singh, et al. (2005). "Differential PsaA-, PspA-, PspC-, and PdB-specific immune responses in a mouse model of pneumococcal carriage." Infect Immun **73**(2): 1006-13.

Palmowski, M., M. Salio, et al. (2002). "The use of HLA class I tetramers to design a vaccination strategy for melanoma patients." Immunol Rev **188**: 155-63.

Pan, E. S., B. A. Diep, et al. (2005). "Population dynamics of nasal strains of methicillin-resistant Staphylococcus aureus--and their relation to community-associated disease activity." J Infect Dis **192**(5): 811-8.

Paniker, J., Ed. (2006). Ananthanarayan and Paniker's Textbook of Microbiology, Orient Longman

Park, S. M., H. J. Ko, et al. (2008). "MyD88 signaling is not essential for induction of antigen-specific B cell responses but is indispensable for protection against Streptococcus pneumoniae infection following oral vaccination with attenuated Salmonella expressing PspA antigen." J Immunol **181**(9): 6447-55.

Paterson, G. K. and T. J. Mitchell (2006). "Innate immunity and the pneumococcus." Microbiology **152**(Pt 2): 285-93.

Paton, J. C. (1996). "The contribution of pneumolysin to the pathogenicity of Streptococcus pneumoniae." Trends Microbiol **4**(3): 103-6.

Pavia, M., A. Bianco, et al. (2009). "Efficacy of pneumococcal vaccination in children younger than 24 months: a meta-analysis." Pediatrics **123**(6): e1103-10.

Pelton, S. I., A. M. Loughlin, et al. (2004). "Seven valent pneumococcal conjugate vaccine immunization in two Boston communities: changes in serotypes and antimicrobial susceptibility among Streptococcus pneumoniae isolates." Pediatr Infect Dis J **23**(11): 1015-22.

Pettigrew, M. M., K. P. Fennie, et al. (2006). "Variation in the presence of neuraminidase genes among Streptococcus pneumoniae isolates with identical sequence types." Infect Immun **74**(6): 3360-5.

Pillai, S., A. Cariappa, et al. (2005). "Marginal zone B cells." Annu Rev Immunol **23**: 161-96.

Pimenta, F. C., E. N. Miyaji, et al. (2006). "Intranasal immunization with the cholera toxin B subunit-pneumococcal surface antigen A fusion protein induces protection against colonization with Streptococcus pneumoniae and has negligible impact on the nasopharyngeal and oral microbiota of mice." Infect Immun **74**(8): 4939-44.

Pollard, A. J., K. P. Perrett, et al. (2009). "Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines." Nat Rev Immunol **9**(3): 213-20.

Pracht, D., C. Elm, et al. (2005). "PavA of Streptococcus pneumoniae modulates adherence, invasion, and meningeal inflammation." Infect Immun **73**(5): 2680-9.

Prendergast, A., J. G. Prado, et al. (2010). "HIV-1 infection is characterized by profound depletion of CD161+ Th17 cells and gradual decline in regulatory T cells." AIDS **24**(4): 491-502.

Preston, J. A. and D. H. Dockrell (2008). "Virulence factors in pneumococcal respiratory pathogenesis." Future Microbiol **3**: 205-21.

Qasi S, W. M., Boshi-Pinto C, Cherian T (2007). Global action plan for the prevention and control of pneumina (gapp): Report of an informal consultation. La Mainaz, Gex: UNICEF/WHO.

Quah, B. J., H. S. Warren, et al. (2007). "Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester." Nat Protoc **2**(9): 2049-56.

Quin, L. R., Q. C. Moore, 3rd, et al. (2007). "Pneumolysin, PspA, and PspC contribute to pneumococcal evasion of early innate immune responses during bacteremia in mice." Infect Immun **75**(4): 2067-70.

Rajewsky, K. (1998). "Burnet's unhappy hybrid." Nature **394**(6694): 624-5.

Rapola, S., V. Jantti, et al. (2000). "Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media." J Infect Dis **182**(4): 1146-52.

Rapola, S., T. Kilpi, et al. (2001). "Antibody response to the pneumococcal proteins pneumococcal surface adhesin A and pneumolysin in children with acute otitis media." Pediatr Infect Dis J **20**(5): 482-7.

Raymond, J., I. Le Thomas, et al. (2000). "Sequential colonization by Streptococcus pneumoniae of healthy children living in an orphanage." J Infect Dis **181**(6): 1983-8.

Regev-Yochay, G., R. Malley, et al. (2008). "In vitro bactericidal activity of Streptococcus pneumoniae and bactericidal susceptibility of Staphylococcus aureus strains isolated from cocolonized versus noncocolonized children." J Clin Microbiol **46**(2): 747-9.

Regev-Yochay, G., M. Raz, et al. (2004). "Nasopharyngeal carriage of Streptococcus pneumoniae by adults and children in community and family settings." Clin Infect Dis **38**(5): 632-9.

Reinert, R. R., P. Paradiso, et al. (2010). "Advances in pneumococcal vaccines: the 13-valent pneumococcal conjugate vaccine received market authorization in Europe." Expert Rev Vaccines **9**(3): 229-36.

Reis e Sousa, C. (2006). "Dendritic cells in a mature age." Nat Rev Immunol **6**(6): 476-83.

Reitano, K. N., S. Kottilil, et al. (2009). "Defective plasmacytoid dendritic cell-NK cell cross-talk in HIV infection." AIDS Res Hum Retroviruses **25**(10): 1029-37.

Ren, B., A. J. Szalai, et al. (2004). "Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface." Infect Immun **72**(1): 114-22.

Ren, B., A. J. Szalai, et al. (2003). "Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of Streptococcus pneumoniae." Infect Immun **71**(1): 75-85.

Richards, L., D. M. Ferreira, et al. (2010). "The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease." Immunobiology **215**(4): 251-63.

Robbins, G. K., J. G. Spritzler, et al. (2009). "Incomplete reconstitution of T cell subsets on combination antiretroviral therapy in the AIDS Clinical Trials Group protocol 384." Clin Infect Dis **48**(3): 350-61.

Robinson, K. A., W. Baughman, et al. (2001). "Epidemiology of invasive Streptococcus pneumoniae infections in the United States, 1995-1998: Opportunities for prevention in the conjugate vaccine era." JAMA **285**(13): 1729-35.

Rodriguez-Barradas, M. C., R. A. Tharapel, et al. (1997). "Colonization by Streptococcus pneumoniae among human immunodeficiency virus-infected adults: prevalence of antibiotic resistance, impact of immunization, and characterization by polymerase chain reaction with BOX primers of isolates from persistent S. pneumoniae carriers." J Infect Dis **175**(3): 590-7.

Roitt, I., J. Brostoff, et al. (2001). Immunology. London, Mosby Elsevier Limited.

Romero-Steiner, S., T. Pilishvili, et al. (2003). "Inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-PsaA antibodies." Clin Diagn Lab Immunol **10**(2): 246-51.

Rowland-Jones, S. L. (1999). "HIV: The deadly passenger in dendritic cells." Curr Biol **9**(7): R248-50.

Roy, M., T. Waldschmidt, et al. (1993). "The regulation of the expression of gp39, the CD40 ligand, on normal and cloned CD4+ T cells." J Immunol **151**(5): 2497-510.

Rubins, J. B. and C. Pomeroy (1997). "Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia." Infect Immun **65**(7): 2975-7.

Rubio, V., T. B. Stuge, et al. (2003). "Ex vivo identification, isolation and analysis of tumor-cytolytic T cells." Nat Med **9**(11): 1377-82.

Rudan, I., C. Boschi-Pinto, et al. (2008). "Epidemiology and etiology of childhood pneumonia." Bull World Health Organ **86**(5): 408-16.

Rudan, I., L. Tomaskovic, et al. (2004). "Global estimate of the incidence of clinical pneumonia among children under five years of age." Bull World Health Organ **82**(12): 895-903.

Rupprecht, T. A., B. Angele, et al. (2007). "Complement C1q and C3 are critical for the innate immune response to Streptococcus pneumoniae in the central nervous system." J Immunol **178**(3): 1861-9.

Rutjens, E., J. Vermeulen, et al. (2008). "Chimpanzee CD4+ T cells are relatively insensitive to HIV-1 envelope-mediated inhibition of CD154 up-regulation." Eur J Immunol **38**(4): 1164-72.

Ryan, K. J., Ed. (2004). Sherris Medical Microbiology, McGraw-Hill.

Sabirov, A. and D. W. Metzger (2006). "Intranasal vaccination of neonatal mice with polysaccharide conjugate vaccine for protection against pneumococcal otitis media." Vaccine **24**(27-28): 5584-92.

Sabirov, A. and D. W. Metzger (2008). "Intranasal vaccination of infant mice induces protective immunity in the absence of nasal-associated lymphoid tissue." Vaccine **26**(12): 1566-76.

Said, E. A., F. P. Dupuy, et al. "Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection." Nat Med **16**(4): 452-9.

Sallusto, F., D. Lenig, et al. (1999). "Two subsets of memory T lymphocytes with distinct homing potentials and effector functions." Nature **401**(6754): 708-12.

Samukawa, T., N. Yamanaka, et al. (2000). "Immune responses to specific antigens of Streptococcus pneumoniae and Moraxella catarrhalis in the respiratory tract." Infect Immun **68**(3): 1569-73.

Scott, J. A., A. Hannington, et al. (1999). "Diagnosis of pneumococcal pneumonia in epidemiological studies: evaluation in Kenyan adults of a serotype-specific urine latex agglutination assay." Clin Infect Dis **28**(4): 764-9.

Sempowski, G. D., C. B. Hicks, et al. (2005). "Naive T cells are maintained in the periphery during the first 3 months of acute HIV-1 infection: implications for analysis of thymus function." J Clin Immunol **25**(5): 462-72.

Seo, J. Y., S. Y. Seong, et al. (2002). "Cross-protective immunity of mice induced by oral immunization with pneumococcal surface adhesin a encapsulated in microspheres." Infect Immun **70**(3): 1143-9.

Shah, P., D. E. Briles, et al. (2009). "Mucosal immunization with polyamine transport protein D (PotD) protects mice against nasopharyngeal colonization with Streptococcus pneumoniae." Exp Biol Med (Maywood) **234**(4): 403-9.

Shaper, M., S. K. Hollingshead, et al. (2004). "PspA protects Streptococcus pneumoniae from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected]." Infect Immun **72**(9): 5031-40.

Sheth, P. M., D. Chege, et al. (2008). "Immune reconstitution in the sigmoid colon after long-term HIV therapy." Mucosal Immunol **1**(5): 382-8.

Shi, Y., K. Agematsu, et al. (2003). "Functional analysis of human memory B-cell subpopulations: IgD+CD27+ B cells are crucial in secondary immune response by producing high affinity IgM." Clin Immunol **108**(2): 128-37.

Shi, Y., T. Yamazaki, et al. (2005). "Regulation of aged humoral immune defense against pneumococcal bacteria by IgM memory B cell." J Immunol **175**(5): 3262-7.

Siddique, M. A., K. E. Hartman, et al. (2006). "Low CD4+ T cell nadir is an independent predictor of lower HIV-specific immune responses in chronically HIV-1-infected subjects receiving highly active antiretroviral therapy." J Infect Dis **194**(5): 661-5.

Sieg, S. F., J. B. Mitchem, et al. (2002). "Close link between CD4+ and CD8+ T cell proliferation defects in patients with human immunodeficiency virus disease and relationship to extended periods of CD4+ lymphopenia." J Infect Dis **185**(10): 1401-16.

Silvestri, G. and M. B. Feinberg (2003). "Turnover of lymphocytes and conceptual paradigms in HIV infection." J Clin Invest **112**(6): 821-4.

Simell, B., T. M. Kilpi, et al. (2002). "Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal capsular polysaccharides in children." J Infect Dis **186**(8): 1106-14.

Simell, B., M. Korkeila, et al. (2001). "Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal surface adhesin a, pneumolysin, and pneumococcal surface protein a in children." J Infect Dis **183**(6): 887-96.

Sleeman, K. L., L. Daniels, et al. (2005). "Acquisition of Streptococcus pneumoniae and nonspecific morbidity in infants and their families: a cohort study." Pediatr Infect Dis J **24**(2): 121-7.

Slifka, M. K., R. Antia, et al. (1998). "Humoral immunity due to long-lived plasma cells." Immunity **8**(3): 363-72.

Soininen, A., H. Pursiainen, et al. (2001). "Natural development of antibodies to pneumococcal capsular polysaccharides depends on the serotype: association with pneumococcal carriage and acute otitis media in young children." J Infect Dis **184**(5): 569-76.

Srivastava, A., P. Henneke, et al. (2005). "The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease." Infect Immun **73**(10): 6479-87.

Stevenson, M. (2003). "HIV-1 pathogenesis." Nat Med **9**(7): 853-60.

Stoscheck, C. M. (1990). "Quantitation of protein." Methods Enzymol **182**: 50-68.

Straw, A. D., A. S. MacDonald, et al. (2003). "CD154 plays a central role in regulating dendritic cell activation during infections that induce Th1 or Th2 responses." J Immunol **170**(2): 727-34.

Strober, W. (2001). "Trypan blue exclusion test of cell viability." Curr Protoc Immunol **Appendix 3:** Appendix 3B.

Subauste, C. S., A. Subauste, et al. (2007). "Role of CD40-dependent down-regulation of CD154 in impaired induction of CD154 in CD4(+) T cells from HIV-1-infected patients." J Immunol **178**(3): 1645-53.

Subauste, C. S., M. Wessendarp, et al. (2004). "Pathogen-specific induction of CD154 is impaired in CD4+ T cells from human immunodeficiency virus-infected patients." J Infect Dis **189**(1): 61-70.

Subauste, C. S., M. Wessendarp, et al. (1999). "CD40-CD40 ligand interaction is central to cell-mediated immunity against Toxoplasma gondii: patients with hyper IgM syndrome have a defective type 1 immune response that can be restored by soluble CD40 ligand trimer." J Immunol **162**(11): 6690-700.

Sun, K., S. L. Salmon, et al. (2007). "Interleukin-12 promotes gamma interferon-dependent neutrophil recruitment in the lung and improves protection against respiratory Streptococcus pneumoniae infection." Infect Immun **75**(3): 1196-202.

Sutherland, J. S., J. M. Young, et al. (2010). "Polyfunctional CD4(+) and CD8(+) T cell responses to tuberculosis antigens in HIV-1-infected patients before and after anti-retroviral treatment." J Immunol **184**(11): 6537-44.

Syrjanen, R. K., T. M. Kilpi, et al. (2001). "Nasopharyngeal carriage of Streptococcus pneumoniae in Finnish children younger than 2 years old." J Infect Dis **184**(4): 451-9.

Tai, S. S. (2006). "Streptococcus pneumoniae protein vaccine candidates: properties, activities and animal studies." Crit Rev Microbiol **32**(3): 139-53.

Talbot, T. R., K. A. Poehling, et al. (2004). "Reduction in high rates of antibiotic-nonsusceptible invasive pneumococcal disease in tennessee after introduction of the pneumococcal conjugate vaccine." Clin Infect Dis **39**(5): 641-8.

Tangye, S. G., Y. J. Liu, et al. (1998). "Identification of functional human splenic memory B cells by expression of CD148 and CD27." J Exp Med **188**(9): 1691-703.

Tejiokem, M. C., I. Gouandjika, et al. (2007). "HIV-infected children living in Central Africa have low persistence of antibodies to vaccines used in the Expanded Program on Immunization." PLoS One **2**(12): e1260.

Titanji, K., A. De Milito, et al. (2006). "Loss of memory B cells impairs maintenance of long-term serologic memory during HIV-1 infection." Blood **108**(5): 1580-7.

Titanji, K., A. Nilsson, et al. (2003). "Low frequency of plasma nerve-growth factor detection is associated with death of memory B lymphocytes in HIV-1 infection." Clin Exp Immunol **132**(2): 297-303.

Trzcinski, K., C. Thompson, et al. (2005). "Antibodies to conserved pneumococcal antigens correlate with, but are not required for, protection against pneumococcal colonization induced by prior exposure in a mouse model." Infect Immun **73**(10): 7043-6.

Trzcinski, K., C. M. Thompson, et al. (2008). "Protection against nasopharyngeal colonization by Streptococcus pneumoniae is mediated by antigen-specific CD4+ T cells." Infect Immun **76**(6): 2678-84.

Tseng, H. J., A. G. McEwan, et al. (2002). "Virulence of Streptococcus pneumoniae: PsaA mutants are hypersensitive to oxidative stress." Infect Immun **70**(3): 1635-9.

Tsigrelis, C., I. M. Tleyjeh, et al. (2008). "Decreases in case-fatality and mortality rates for invasive pneumococcal disease in Olmsted County, Minnesota, during 1995-2007: a population-based study." Clin Infect Dis **47**(11): 1367-71.

Tsunemi, S., T. Iwasaki, et al. (2005). "Relationship of CD4+CD25+ regulatory T cells to immune status in HIV-infected patients." AIDS **19**(9): 879-86.

Tzianabos, A. O., R. W. Finberg, et al. (2000). "T cells activated by zwitterionic molecules prevent abscesses induced by pathogenic bacteria." J Biol Chem **275**(10): 6733-40.

USAID. (2010). "HIV/AIDS Health profile-Malawi." Retrieved 9 March, 2011, from www.usaid.gov/our-work/global-health/aids/..../malawi-profile.pdf.

Van Damme, W., Kober, K., Laga, M., (2006). "The real challenges for scaling up ART in sub-Saharan Africa." AIDS **20**: 653-656.

van Kooten, C. and J. Banchereau (2000). "CD40-CD40 ligand." J Leukoc Biol **67**(1): 2-17.

van Rossum, A. M., E. S. Lysenko, et al. (2005). "Host and bacterial factors contributing to the clearance of colonization by Streptococcus pneumoniae in a murine model." Infect Immun **73**(11): 7718-26.

Vesikari, T., J. Wysocki, et al. (2009). "Immunogenicity of the 10-valent pneumococcal non-typeable Haemophilus influenzae protein D conjugate vaccine (PHiD-CV) compared to the licensed 7vCRM vaccine." Pediatr Infect Dis J **28**(4 Suppl): S66-76.

Vinuesa, C. G., D. M. Sze, et al. (2003). "Recirculating and germinal center B cells differentiate into cells responsive to polysaccharide antigens." Eur J Immunol **33**(2): 297-305.

Virolainen, A., W. Russell, et al. (2000). "Human antibodies to pneumococcal surface protein A in health and disease." Pediatr Infect Dis J **19**(2): 134-8.

Vollers, S. S. and L. J. Stern (2008). "Class II major histocompatibility complex tetramer staining: progress, problems, and prospects." Immunology **123**(3): 305-13.

Vrisekoop, N., R. van Gent, et al. (2008). "Restoration of the CD4 T cell compartment after long-term highly active antiretroviral therapy without phenotypical signs of accelerated immunological aging." J Immunol **181**(2): 1573-81.

Walker, M. R., D. J. Kasprowicz, et al. (2003). "Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells." J Clin Invest **112**(9): 1437-43.

Wallace, P. K., J. D. Tario, Jr., et al. (2008). "Tracking antigen-driven responses by flow cytometry: monitoring proliferation by dye dilution." Cytometry A **73**(11): 1019-34.

Walport, M. J. (2001). "Complement. First of two parts." N Engl J Med **344**(14): 1058-66.

Walport, M. J. (2001). "Complement. Second of two parts." N Engl J Med **344**(15): 1140-4.

Wang, X., Z. Cao, et al. (2010). "Association of mycobacterial antigen-specific CD4(+) memory T cell subsets with outcome of pulmonary tuberculosis." J Infect **60**(2): 133-9.

Warrington, K. J., A. N. Vallejo, et al. (2003). "CD28 loss in senescent CD4+ T cells: reversal by interleukin-12 stimulation." Blood **101**(9): 3543-9.

Watson, D. A. and D. M. Musher (1999). "A brief history of the pneumococcus in biomedical research." Semin Respir Infect **14**(3): 198-208.

Weinberger, D. M., R. Dagan, et al. (2008). "Epidemiologic evidence for serotype-specific acquired immunity to pneumococcal carriage." J Infect Dis **197**(11): 1511-8.

Weiser, J. N. (2009). "The pneumococcus: why a commensal misbehaves." J Mol Med **88**(2): 97-102.

Weiser, J. N., D. Bae, et al. (2003). "Antibody-enhanced pneumococcal adherence requires IgA1 protease." Proc Natl Acad Sci U S A **100**(7): 4215-20.

Weiser, J. N. and M. H. Nahm (2008). Immunity to Extracellular Bacteria. Fundamental Immunology. W. E. Paul, Lippincott Williams & Wilkins.

Weiss, L., V. Donkova-Petrini, et al. (2004). "Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients." Blood **104**(10): 3249-56.

Weller, S., M. C. Braun, et al. (2004). "Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire." Blood **104**(12): 3647-54.

Wherry, E. J., V. Teichgraber, et al. (2003). "Lineage relationship and protective immunity of memory CD8 T cell subsets." Nat Immunol **4**(3): 225-34.

Whitney, C. G. (2005). "Impact of conjugate pneumococcal vaccines." Pediatr Infect Dis J **24**(8): 729-30.

Whitney, C. G., M. M. Farley, et al. (2003). "Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine." N Engl J Med **348**(18): 1737-46.

Whitney, C. G., T. Pilishvili, et al. (2006). "Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study." Lancet **368**(9546): 1495-502.

WHO. (2006). "Country Health System Fact Sheet 2006-Malawi." Retrieved 20 April 2010, from http://www.afro.who.int/home/countries/fact_sheets/malawi.pdf.

WHO. (2007). "World health statistics." Retrieved 20 Nov 2008, from http://www.who.int/whosis/whostat2007.pdf

WHO. (2008a). "Malawi." Retrieved 2 December 2008, from http://www.who.int/countries/mwi/en/.

WHO. (2008b). "Malawi: Epidemiological Country Profile on HIV and AIDS." Retrieved 20 April 2010, from http://apps.who.int/globalatlas/predefinedReports/EFS2008/short/EFSCountryProfiles2008_MW.pdf.

WHO (2008c). 23-Valent pneumococcal polysaccharide
vaccine: WHO position paper. **83:** 373-84.

WHO (2008d). 23-valent pneumococcal polysaccharide vaccine:WHO position paper. Weekly epidemiological record: 373–384.

WHO. (2009). "New HIV recommendations to improve health, reduce infections and save lives. ." Retrieved 6 March 2011, from http://www.who.int/mediacentre/news/releases/2009/world_aids_20091130/en/index.html.

Wiley, R. D. and S. Gummuluru (2006). "Immature dendritic cell-derived exosomes can mediate HIV-1 trans infection." Proc Natl Acad Sci U S A **103**(3): 738-43.

Wilkinson, K. A., R. Seldon, et al. (2009). "Dissection of regenerating T-Cell responses against tuberculosis in HIV-infected adults sensitized by Mycobacterium tuberculosis." Am J Respir Crit Care Med **180**(7): 674-83.

WorldVision. (2008). "Map Malawi." Retrieved 2 December 2009), from www.30hourfamine.org/.../asset/53_map_malawi.jpg.

Wu, C. Y., J. R. Kirman, et al. (2002). "Distinct lineages of T(H)1 cells have differential capacities for memory cell generation in vivo." Nat Immunol **3**(9): 852-8.

Wu, H. Y., M. H. Nahm, et al. (1997). "Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with Streptococcus pneumoniae." J Infect Dis **175**(4): 839-46.

Wu, L. and V. N. KewalRamani (2006). "Dendritic-cell interactions with HIV: infection and viral dissemination." Nat Rev Immunol **6**(11): 859-68.

Wysocki, J., J. C. Tejedor, et al. (2009). "Immunogenicity of the 10-valent pneumococcal non-typeable Haemophilus influenzae protein D conjugate vaccine (PHiD-CV) when coadministered with different neisseria meningitidis serogroup C conjugate vaccines." Pediatr Infect Dis J **28**(4 Suppl): S77-88.

Xin, W., Y. Li, et al. (2009). "PspA family fusion proteins delivered by attenuated Salmonella enterica serovar Typhimurium extend and enhance protection against Streptococcus pneumoniae." Infect Immun **77**(10): 4518-28.

Xin, W., S. Y. Wanda, et al. (2008). "Analysis of type II secretion of recombinant pneumococcal PspA and PspC in a Salmonella enterica serovar Typhimurium vaccine with regulated delayed antigen synthesis." Infect Immun **76**(7): 3241-54.

Xu, W., P. A. Santini, et al. (2009). "HIV-1 evades virus-specific IgG2 and IgA responses by targeting systemic and intestinal B cells via long-range intercellular conduits." Nat Immunol **10**(9): 1008-17.

Yamamoto, M., L. S. McDaniel, et al. (1997). "Oral immunization with PspA elicits protective humoral immunity against Streptococcus pneumoniae infection." Infect Immun **65**(2): 640-4.

Ye, P., P. B. Garvey, et al. (2001). "Interleukin-17 and lung host defense against Klebsiella pneumoniae infection." Am J Respir Cell Mol Biol **25**(3): 335-40.

Yellin, M. J., K. Sippel, et al. (1994). "CD40 molecules induce down-modulation and endocytosis of T cell surface T cell-B cell activating molecule/CD40-L. Potential role in regulating helper effector function." J Immunol **152**(2): 598-608.

Yoo, J., H. Chen, et al. (1996). "Altered cytokine production and accessory cell function after HIV-1 infection." J Immunol **157**(3): 1313-20.

Yue, F. Y., C. M. Kovacs, et al. (2005). "Preferential apoptosis of HIV-1-specific CD4+ T cells." J Immunol **174**(4): 2196-204.

Yuste, J., M. Botto, et al. (2005). "Additive inhibition of complement deposition by pneumolysin and PspA facilitates Streptococcus pneumoniae septicemia." J Immunol **175**(3): 1813-9.

Zar, H. J. (2004). "Pneumonia in HIV-infected and HIV-uninfected children in developing countries: epidemiology, clinical features, and management." Curr Opin Pulm Med **10**(3): 176-82.

Zhang, J. R., K. E. Mostov, et al. (2000). "The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells." Cell **102**(6): 827-37.

Zhang, Q., K. Arnaoutakis, et al. (2004). "Mucosal immune responses to capsular pneumococcal polysaccharides in immunized preschool children and controls with similar nasal pneumococcal colonization rates." Pediatr Infect Dis J **23**(4): 307-13.

Zhang, Q., L. Bagrade, et al. (2007). "Low CD4 T cell immunity to pneumolysin is associated with nasopharyngeal carriage of pneumococci in children." J Infect Dis **195**(8): 1194-202.

Zhang, Q., J. Bernatoniene, et al. (2006). "Serum and mucosal antibody responses to pneumococcal protein antigens in children: relationships with carriage status." Eur J Immunol **36**(1): 46-57.

Zhang, Q., S. Choo, et al. (2002). "Immune responses to novel pneumococcal proteins pneumolysin, PspA, PsaA, and CbpA in adenoidal B cells from children." Infect Immun **70**(10): 5363-9.

Zhang, Q., R. Lakshman, et al. (2001). "Primary and booster mucosal immune responses to meningococcal group A and C conjugate and polysaccharide vaccines administered to university students in the United Kingdom." Infect Immun **69**(7): 4337-41.

Zhang, R., C. J. Fichtenbaum, et al. (2004). "CD40 ligand dysregulation in HIV infection: HIV glycoprotein 120 inhibits signaling cascades upstream of CD40 ligand transcription." J Immunol **172**(4): 2678-86.

Zhang, R., J. D. Lifson, et al. (2006). "Failure of HIV-exposed CD4+ T cells to activate dendritic cells is reversed by restoration of CD40/CD154 interactions." Blood **107**(5): 1989-95.

Zhang, Z., T. B. Clarke, et al. (2009). "Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice." J Clin Invest **119**(7): 1899-909.

Zysk, G., L. Bejo, et al. (2000). "Induction of necrosis and apoptosis of neutrophil granulocytes by Streptococcus pneumoniae." Clin Exp Immunol **122**(1): 61-6.

APPENDICES

APPENDIX I

WHO CLINICAL STAGING OF HIV/AIDS FOR ADULTS AND ADOLESCENTS

Primary HIV infection

- Asymptomatic
- Acute retroviral syndrome

Clinical stage 1

- Asymptomatic
- Persistent generalized lymphadenopathy (PGL)

Clinical stage 2

- Moderate unexplained weight loss (<10% of presumed or measured body weight)
- Recurrent respiratory tract infections (RTIs, sinusitis, bronchitis, otitis media, pharyngitis)
- Herpes zoster
- Angular cheilitis
- Recurrent oral ulcerations
- Papular pruritic eruptions
- Seborrhoeic dermatitis
- Fungal nail infections of fingers

Clinical stage 3

Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations

- Severe weight loss (>10% of presumed or measured body weight)
- Unexplained chronic diarrhoea for longer than one month
- Unexplained persistent fever (intermittent or constant for longer than one month)
- Oral candidiasis
- Oral hairy leukoplakia
- Pulmonary tuberculosis (TB) diagnosed in last two years
- Severe presumed bacterial infections (e.g. pneumonia, empyema, pyomyositis, bone or
- joint infection, meningitis, bacteraemia)
- Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis

Conditions where confirmatory diagnostic testing is necessary

- Unexplained anaemia (< 8 g/dl), and or neutropenia (<500/mm3) and or
- thrombocytopenia (<50 000/ mm3) for more than one month

Clinical stage 4

Conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations

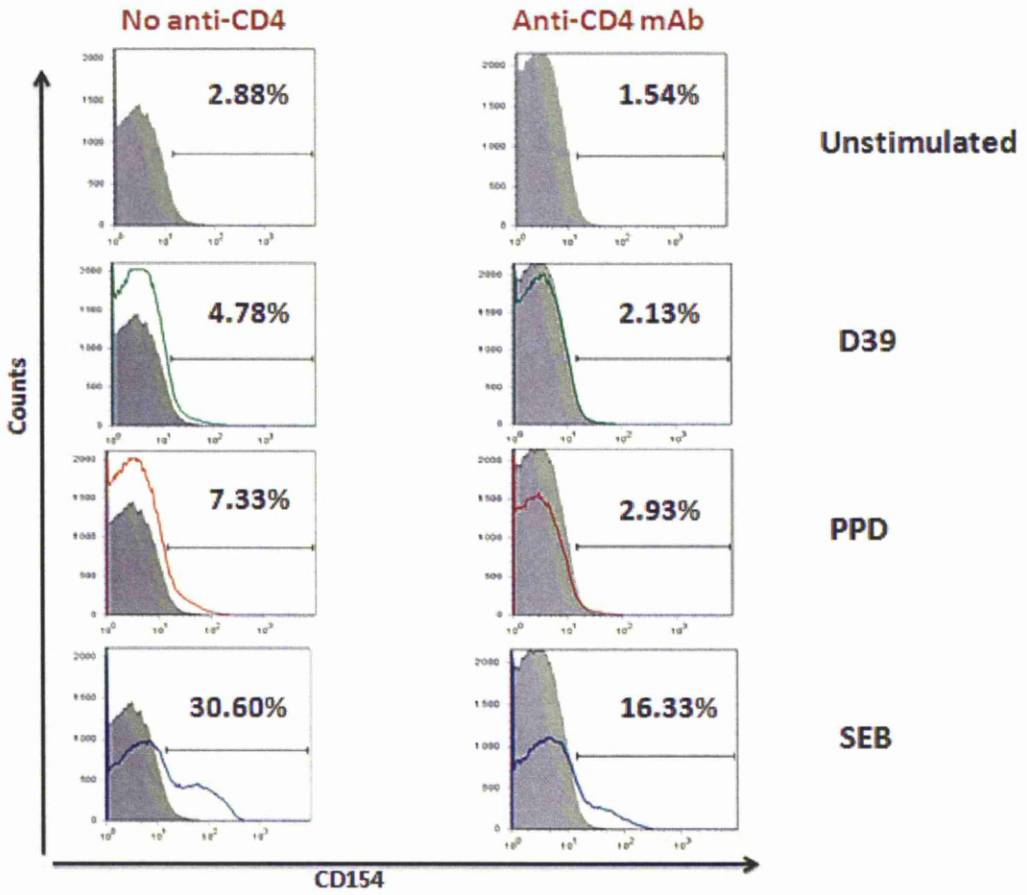
- HIV wasting syndrome
- Pneumocystis pneumonia
- Recurrent severe or radiological bacterial pneumonia

- Chronic herpes simplex infection (orolabial, genital or anorectal of more than one month's duration)
- Oesophageal candidiasis
- Extrapulmonary TB
- Kaposi's sarcoma
- Central nervous system (CNS) toxoplasmosis
- HIV encephalopathy

Conditions where confirmatory diagnostic testing is necessary

- Extrapulmonary cryptococcosis including meningitis
- Disseminated non-tuberculous mycobacteria infection
- Progressive multifocal leukoencephalopathy (PML)
- Candida of trachea, bronchi or lungs
- Cryptosporidiosis
- Isosporiasis
- Visceral herpes simplex infection
- Cytomegalovirus (CMV) infection (retinitis or of an organ other than liver, spleen or lymph nodes)
- Any disseminated mycosis (e.g. histoplasmosis, coccidiomycosis, penicilliosis)
- Recurrent non-typhoidal salmonella septicaemia
- Lymphoma (cerebral or B cell non-Hodgkin)
- Invasive cervical carcinoma
- Visceral leishmaniasis

Appendix II



Appendix II | CD4 ligation and CD154 expression. 2 x10^6 freshly isolated PBMCs were plated in a 96-well plate in 200 ul 1X sterile PBS. An anti-CD4 domain 1 blocking antibody (final concentration 10ug/ml [clone QS4120; Ancell, Bayport, MN]) was added to appropriate wells and the plate was incubated for 1h at 4^0C as described elsewhere (Zhang, Fichtenbaum et al. 2004; Rutjens, Vermeulen et al. 2008). Thereafter, the cells were washed twice with 1ml sterile 1X PBS at 700 g for 7 minutes and resuspended in RPMI 1640 medium supplemented with 2% heat inactivated AB human serum (National Blood Services, Blantyre, Malawi), 2 mM L-glutamine, 100 U/ml penicillin, 20 mM HEPES buffer and 100 ug/ml streptomycin (Sigma). An anti-CD154 antibody was introduced into the cell culture immediately before stimulation and cells cultured for 18 hours.

Review

Potential role for mucosally active vaccines against pneumococcal pneumonia

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Pneumococcal pneumonia is a life-threatening disease with high mortality and morbidity among children under 5 years of age, the elderly and immunocompromised individuals worldwide. Protection against pneumococcal pneumonia relies on successful regulation of colonisation in the nasopharynx and a brisk alveolar macrophage-mediated immune response in the lung. Therefore, enhancing pulmonary mucosal immunity (which includes a combination of innate, humoral and cell-mediated immunity) through mucosal vaccination might be the key to prevention of pneumococcal infection. Current challenges include a lack of information in humans on mucosal immunity against pneumococci and a lack of suitable adjuvants for new vaccines. Data from mouse models, however, suggest that mucosally active vaccines will enhance mucosal and systemic immunity for protection against pneumococcal infection.

Prevention of pneumococcal pneumonia: current strategies

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive aerobic commensal bacterium which forms part of the normal flora in the nasopharynx [1]. The pneumococcus can evade the immune system through a combination of surface expressed and secreted virulence factors to cause mucosal diseases such as otitis media, sinusitis and pneumonia, as well as systemic diseases such as bacteraemia and meningitis [1,2]. These diseases, collectively termed pneumococcal disease, can be classified as invasive or non-invasive disease. Otitis media, sinusitis and non-bacteraemic pneumococcal pneumonia are examples of non-invasive disease which are confined to the mucosal surface, whereas bacteraemic pneumonia, bacteraemia and meningitis are examples of invasive disease. Bacteraemic pneumococcal pneumonia, defined as having pneumonia and a positive blood culture [3], is more common in HIV-infected patients. Invasive pneumococcal disease is thought to progress from colonisation to bacteraemia, with or without pneumonia, only a minority of cases developing meningitis (**Figure 1**).

Pneumonia accounts for 19% of all under 5 year old deaths worldwide, which makes it the most deadly infectious illness for this age group [4]. The pneumococcus is the leading cause of pneumonia in children and it has been reported to cause over 50% of severe pneumonia cases in Africa [4]. Pneumococcal disease is most prevalent in the young and the elderly, but is also very common among HIV-infected individuals, who are 20–40 times more likely than uninfected adults to suffer from this illness [5].

Pneumococcal pneumonia is treatable using antibiotic therapy. However, where treatment is delayed or unavailable mortality is high [5]. Previously, the developing world had focused on *treating* pneumococcal disease rather than *preventing* it, but with the current increase in antibiotic resistance and the HIV pandemic, it is widely accepted that prevention is the key to minimising the disease burden [5].

Vaccination offers the most efficient and cost-effective method of preventing this disease. However, there are more than 90 pneumococcal serotypes which make development of a vaccine to provide universal protection a big challenge. There are two formulations of pneumococcal vaccines that have been licensed thus far: polysaccharide vaccines (PPVs) and protein conjugate vaccines (PCVs). The 23-valent pneumococcal polysaccharide vaccine, which contains purified capsular polysaccharide antigens from 23 serotypes, offers some protection against invasive pneumococcal disease in adults but is not effective in either children less than 2 years

Glossary

Colonisation: establishment of bacteria in the upper respiratory tract without causing overt disease.
Compartmentalisation: in the context of this article, the regional sequestration of a specific function within the immune system. Local immune responses within the body (site of infection) are often functionally independent from systemic immunity.
Complement system: a cascade of plasma proteins that act together in defence against extracellular bacteria by coating bacteria (opsonisation) or killing pathogens directly.
Mucosal immunity: part of the immune system that is responsible for protection against pathogens, commensals and other non-microbial antigens restricted to the mucous membranes of the gastrointestinal, respiratory and urinogenital tracts, i.e. the major sites of antigen/pathogen entry into the body.
Opsonisation: alteration of surfaces of pathogens by immune molecules in such a manner that the pathogens are more easily recognised by immune cells.
Opsonophagocytosis: the process whereby bacteria are altered for optimal interaction with phagocytes and then engulfed.
Systemic immunity: the general immune system responsible for protection against pathogens, commensals and other non-microbial antigens; it is not confined to the site of infection or antigen entry.

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0966-842X/$ – see front matter doi:10.1016/j.tim.2009.12.001 Available online 22 December 2009

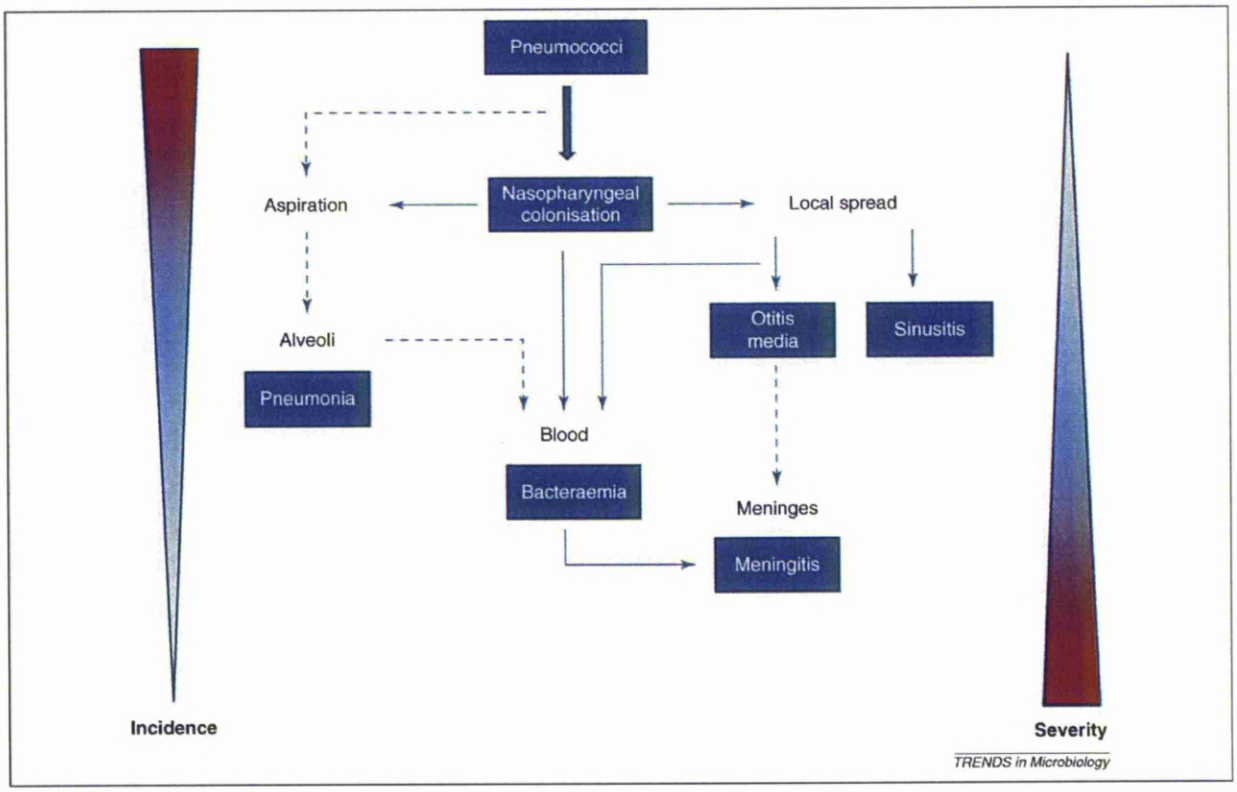

Figure 1. Diseases caused by *Streptococcus pneumoniae*. Pneumococci colonise the nasopharynx, evade host immunity and spread to the middle ear, sinus, lower respiratory tract, blood and meninges. Pneumococci cause otitis media in the middle ear, sinusitis in the sinus, pneumonia in the lower respiratory tract, bacteraemia in blood and meningitis in the meninges. The incidences of different types of pneumococcal infection are inversely related to the severity of disease: otitis media is the most common but the least severe. Redrawn and redesigned with permission from Ref. [2].

of age or immunocompromised adults [6]. PCVs, which contain purified capsular polysaccharides conjugated to a carrier protein, offer protection against both pneumonia and invasive disease in children [7] and immunocompromised adults (French *et al.* unpublished). The currently licensed 7-valent conjugate vaccine (containing 7 capsular polysaccharides conjugated to a diphtheria CRM197 protein) is being used as part of childhood immunisation programmes in several countries but others are waiting for the licensing of 10-valent and 13-valent vaccines. The disadvantages of PCVs are that they are expensive, have limited serotype coverage, can be associated with an increase in disease caused by serotypes not included in the vaccine and are less effective against radiological pneumonia (20–37% efficacy) [7,8] than against invasive disease (77–83% efficacy) [7]. In African children, PCVs appear to provide no protection to unvaccinated children (herd immunity) and is not very effective against colonisation (39% against vaccine serotypes, 0% against all serotypes) [9].

There are several key developments that would result in a breakthrough in the global control of pneumococcal disease. Use of the PCV is an important landmark [8] but the use of conserved proteins in a universal vaccine would allow a single vaccine to be deployed in all geographical regions without regard to the prevalent serotype patterns. In addition, the development of mucosally active vaccines might reduce mucosal disease including pneumonia and otitis media, but this will require the identification of safe and effective mucosal adjuvants for successful vaccine delivery.

This review will focus on recent advances in our understanding of mucosal immunity relevant to pneumococcal infection and, in particular, the critical immune responses that must be augmented by new vaccines.

Mucosal immunity against *Streptococcus pneumonia*

Host response against pneumococcal colonisation

Pneumococcal colonisation of the upper respiratory tract precedes infection of the lower respiratory tract, but is normally asymptomatic and not usually followed by disease [2]. The local host immune response plays an important role in regulating the containment of pathogens within the nasopharyngeal cavity [2]. A brisk local host immune response to *S. pneumoniae* involving phagocytes (neutrophils and macrophages), B cells (antibodies against pneumococcal polysaccharides and proteins) and T cells rapidly eliminates colonisation, whereas a poor mucosal immune response results in protracted colonisation [2]. Both innate and adaptive immunity play a role in these host defence responses against *S. pneumoniae*.

Innate immune response during colonisation

Innate factors (including C-reactive protein, CRP) play a crucial role in the host defence against colonisation with *S.*

pneumoniae. CRP is an acute-phase protein which is mainly found in serum and elevated during inflammation [10]. It has also been detected in the upper respiratory tract of healthy individuals and is also elevated during inflammation [11]. The concentration of CRP is lower in the respiratory tract than in serum but is sufficient to contribute to innate immunity, and is locally produced by epithelial cells [11,12]. CRP has several functions in relation to cell-surface phosphorylcholine-expressing bacteria (such as *S. pneumoniae* and *Haemophilus influenzae*), which includes activation of complement by the classical pathway, enhancing opsonisation and inhibition of the attachment of bacteria to epithelial cells [12]. These functions help in clearance of colonising pneumococci from the upper respiratory tract, which if not cleared might transmigrate across endothelial and epithelial monolayers in a process called pericellular tissue invasion [13].

It is known that complement plays a role in protection against pneumococcal infection through the promotion of opsonophagocytosis [14,15]. However, there are still conflicting data on the role of complement in protection against colonisation. Data from a co-colonisation mice model (of *S. pneumoniae* and *H. influenzae*) suggest that successful clearance of pneumococci in the nasopharynx resulted from opsonisation by complement, followed by phagocytosis by neutrophils which were recruited to the mucosal surface [16]. In a different model, C3 (a protein that plays a central role in activation of the complement system) was essential within the lungs for an optimal immune response against *S. pneumoniae* and subsequently played an important role systemically [17]. In contrast, another murine colonisation model showed that C3-deficient mice lacking functional complement system cleared pneumococcal colonisation at the same rate as wild type mice [18]. It is important to take note that murine models vary according to the genetic background of the mouse and the serotype of pneumococcus used, and hence they might not always produce similar results.

The innate response includes cellular responses from neutrophils and macrophages. It has been demonstrated in murine models that pneumococcal colonisation of the upper respiratory tract triggers an acute inflammatory response characterised by a robust influx of neutrophils into the lumen of the paranasal spaces [18,19] and release of cytokines (tumour necrosis factor alpha, TNF-α) and chemokines (interleukin 8, IL-8) [20]. This acute inflammatory response is ineffective at controlling initial mucosal colonisation [19], but it enhances the adaptive immune response and subsequent bacterial clearance [21].

Adaptive immune response during colonisation

Nasopharyngeal colonisation stimulates the production of secretory IgA antibodies (the predominant immunoglobulin class in human external secretions) and serum IgG [22]. However, it is unclear whether these antibodies are protective against colonisation. Many isolates of *S. pneumoniae* secrete a zinc metalloprotease which inactivates IgA1 (a subclass of IgA). Furthermore, the cleaved IgA1 fragment might assist translocation of the opsonised bacteria across the host respiratory epithelium [23]. By contrast, increased concentrations of serotype-specific antibodies against pneumococcal polysaccharides [24,25] and antibodies against pneumococcal proteins in serum and saliva [26] have been correlated with increased protection against carriage. These antibodies opsonise pneumococci, making it easier for phagocytes to recognise, ingest and clear bacteria from the respiratory tract [27]. This has long been thought to be the primary main mechanism for protection against pneumococcal colonisation.

Box 1. The role of pneumolysin in pneumonia and bacteraemia

Pneumolysin is a potent pore-forming cytotoxin produced by almost all pneumococcal isolates, and a proven virulence factor. Animal studies show that this protein is required for the development of pneumonia and bacteraemia. Data suggests that *S. pneumoniae* requires pneumolysin to successfully survive in both the upper and lower respiratory tracts [1,78,79]. The cytotoxin is essential for pneumococcus to translocate from the lungs to the bloodstream [1,78–80]. It is also required for bacterial survival in blood: pneumolysin-expressing pneumococci are associated with high bacteraemia and severe infection [79,80]. By contrast, pneumolysin-deficient pneumococci result in low bacteraemia (often chronic) with minimal severity in animal models [81].

Several recent studies suggest that other mechanisms of protection against pneumococcal carriage are required, in addition to antibody-mediated immunity. Firstly, the course of an experimental colonisation is not affected in mice that are unable to produce pneumococcal-specific antibody [28]. Secondly, the adaptive immune response is enhanced in the presence of pneumolysin and neutrophils. Pneumolysin, a pore-forming cytotoxin is a critical pathogenic factor of *S. pneumoniae* (**Box 1**). The interaction of pneumolysin and neutrophils promotes delivery and release of pneumococcal-specific antigens to the nasal associated lymphoid tissues, a process that is impaired in either neutrophil- or pneumolysin-deficient conditions [21]. Impaired antigen delivery is associated with prolonged nasopharyngeal colonisation [21]. Finally, mice lacking the ability to induce a cell-mediated immunity owing to the absence of appropriate molecules to present antigens to $CD4^+$ T cells (MHC-II knockout mice) show prolonged carriage, suggesting an important role for $CD4^+$ T cells rather than antibody-mediated immunity [29] (for the antigen presentation process, see **Figure 2**).

Studies have shown that immunity to pneumococcal colonisation is mediated by a specific subset of $CD4^+$ T cells (Th17) which produce IL-17A [30–34]. Malley *et al.* showed that blocking IL-17A in mice models reduced immunity to pneumococcal colonisation following intranasal immunisation with cell wall polysaccharide [33]. In another study by the same group, they showed that IL-17A expression in peripheral blood samples from immunised mice was associated with protection *in vivo* against pneumococcal carriage [32].

IL-17A-mediated protection against pneumococcal colonisation results in recruitment of neutrophils into the upper airway lumen to clear bacterium [32,34]. Recently, Zhang *et al.* showed that primary challenge with pneumococci in mice generates $CD4^+$ T cell memory, resulting in enhanced Th17-mediated recruitment of neutrophils after secondary pneumococcal challenge [34]. These neutrophils were shown to contribute to early bacterial clearance

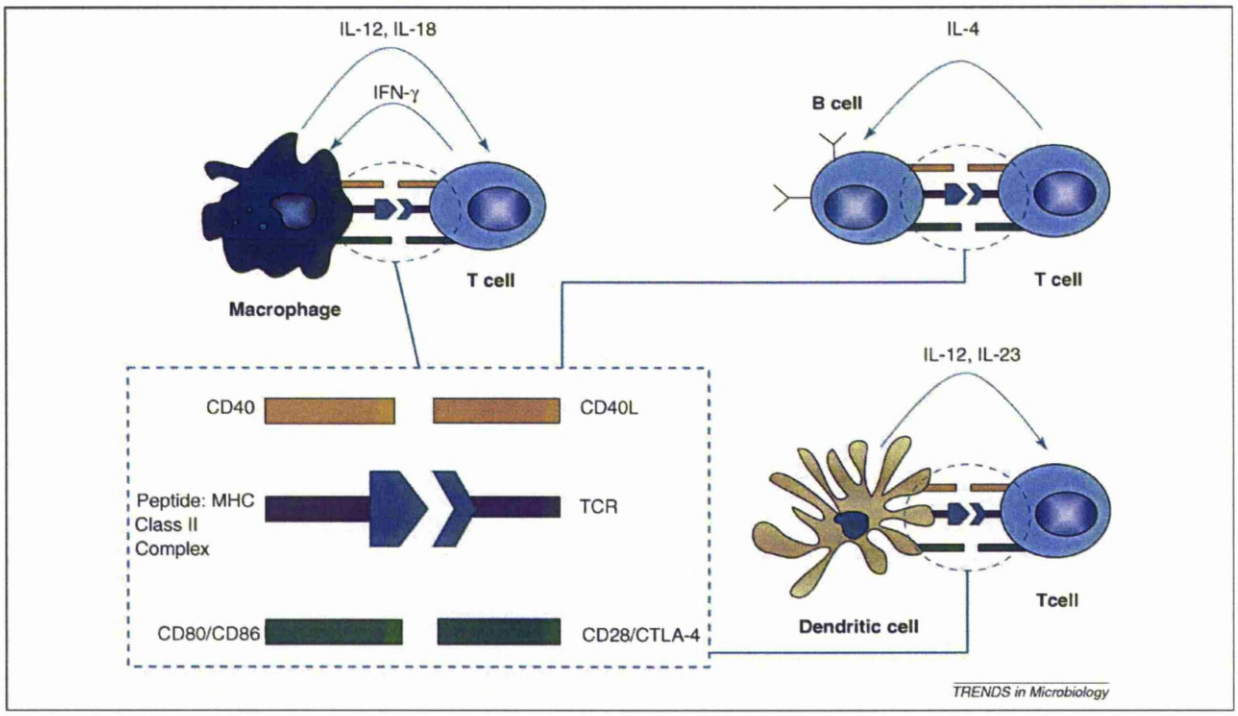

Figure 2. Induction of cell-mediated immune response by $CD4^+$ T cells. There are three signals that are important during T cell activation: antigen presentation (TCR:Peptide-MHC class II ligation), co-stimulation (CD40:CD40L and CD80/CD86:CD28) and polarising signals (cytokine milieu). Professional antigen-presenting cells (dendritic cells, B cells and macrophages) present antigens to T cells in the context of MHC class II via the TCR (T cell receptor). This induces upregulation of CD40L and CD28 on the T cells, which bind to their receptors CD40 and CD80/CD86, respectively, on APCs, in a process called co-stimulation. These events lead to the production of polarising cytokines by APCs which include IL-12 from macrophages and dendritic cells, and IL-23 from dendritic cells. The polarising cytokines are important because they dictate the fate of T cells on whether to differentiate into Th1, Th2 or Th17.

following secondary challenge [34]. However, it is still not clear whether Th17 cells are involved in immunity against human pneumococcal colonisation.

Host response against pneumococcal pneumonia

Post colonisation events leading to pneumonia

If pneumococci colonising the human nasopharynx are aspirated into the distal airways and alveolar air spaces, they will interact with pulmonary defence mechanisms. The bacteria will either be cleared or cause disease. Excessive replication of the bacteria in the alveoli triggers infiltration of immune cells which – if not properly regulated – impairs gas exchange resulting in the clinical syndrome of pneumonia.

The process of bacterial clearance in the lung is a highly regulated process – an excessive response might potentially lead to tissue damage, whereas a weak response leads to exponential growth of the pathogens. The primary host immune defence against small numbers of pneumococci during early infection is phagocytosis [27] which is enhanced through opsonisation by immunoglobulin and complement [19], in a process called opsonophagocytosis. The mechanism of host defence against pneumococci during late infection is different. It involves multiple immune cells and combination of innate and adaptive immunity.

Early infection in the lung

Alveolar macrophages are the first cells that combat pneumococci during early infection [35] and the main cell population that mediates mucosal responses in the lower airways [36,37]. Approximately 90% of cells found in bronchoalveolar lavage fluid of healthy volunteers are macrophages [37]. It has been suggested that during early infection (where the bacterial load is low), resident alveolar macrophages are capable of multiple episodes of phagocytosis and killing [38]. This helps to clear the bacteria without recruitment of inflammatory cells such as neutrophils, and hence maintaining a low inflammatory state in the lung [38]. Early clearance of pneumococci through alveolar macrophage phagocytosis probably prevents bacteria–dendritic cell interaction, which in turn limits the initiation of T cell-mediated inflammatory responses in the lymph node [37].

It is still unclear whether antigen presentation occurs in the lung, in the draining lymph nodes, or both, and whether alveolar macrophages are part of this antigen presentation process. There is data *in vitro* to suggest that alveolar macrophages are able to present antigens to T cells, although less effectively than other antigen-presenting cells (APCs) [39]. Alveolar macrophages might induce antigen-specific unresponsiveness in $CD4^+$ T cells as a result of antigen recognition in the absence of co-stimulation [39] (for the antigen presentation process, see **Figure 2**).

Late infection in the lung

When the alveolar bacterial load rises above a critical threshold, alveolar macrophages cease to perform effective

opsonophagocytosis and produce an increased proinflammatory cytokine response dominated by TNF-α and IL-8 [38]. The presence of TNF-α is not a prerequisite for pulmonary anti-pneumococcal responses, because successful clearance of the bacteria can occur independent of this cytokine – but it is beneficial during systemic infection [40]. Inflamed epithelial cells enhance neutrophil recruitment into the lung by secretion of IL-8 [41], both as a direct result of pneumococcal binding to epithelial receptors and in response to macrophage proinflammatory signalling.

When a proinflammatory signal (TNF-α and/or IL-8) is produced in the alveolus, there is upregulation of adherence molecules on endothelial cells which bind to their receptors on neutrophils. This results in rolling of neutrophils along the endothelial wall and transmigration into the alveolar space in a process called chemotaxis [42]. Neutrophils then become the major immune cell population responsible for pneumococcal clearance in the lung [43].

T cells are also recruited in high numbers to the lung in late infection – the peak of T cell infiltration in the lung during intranasal pneumococcal infection in mice *in vivo* coincided with the phase when bacterial growth ceased [44]. The recruited T cells are predominantly of the effector memory phenotype and potentially secrete interferon gamma IFN-γ to activate alveolar macrophages. The actual mechanisms on the role played by T cells are still not clear, but we have hypothesised some of the possible pathways in Figure 3. In addition, it has been shown that T cells expressing the gammadelta receptor (γδ T cells) act as regulators of alveolar macrophages and pulmonary dendritic cells during the resolution of pneumococcus-mediated lung inflammation [45]. Cytoxicity mediated by γδ T cells helps restore mononuclear phagocyte numbers to homeostatic levels, and hence preventing excessive inflammation in the lung [45].

Following clearance of pneumococci from the lungs, neutrophils, some macrophages and T cells undergo rapid

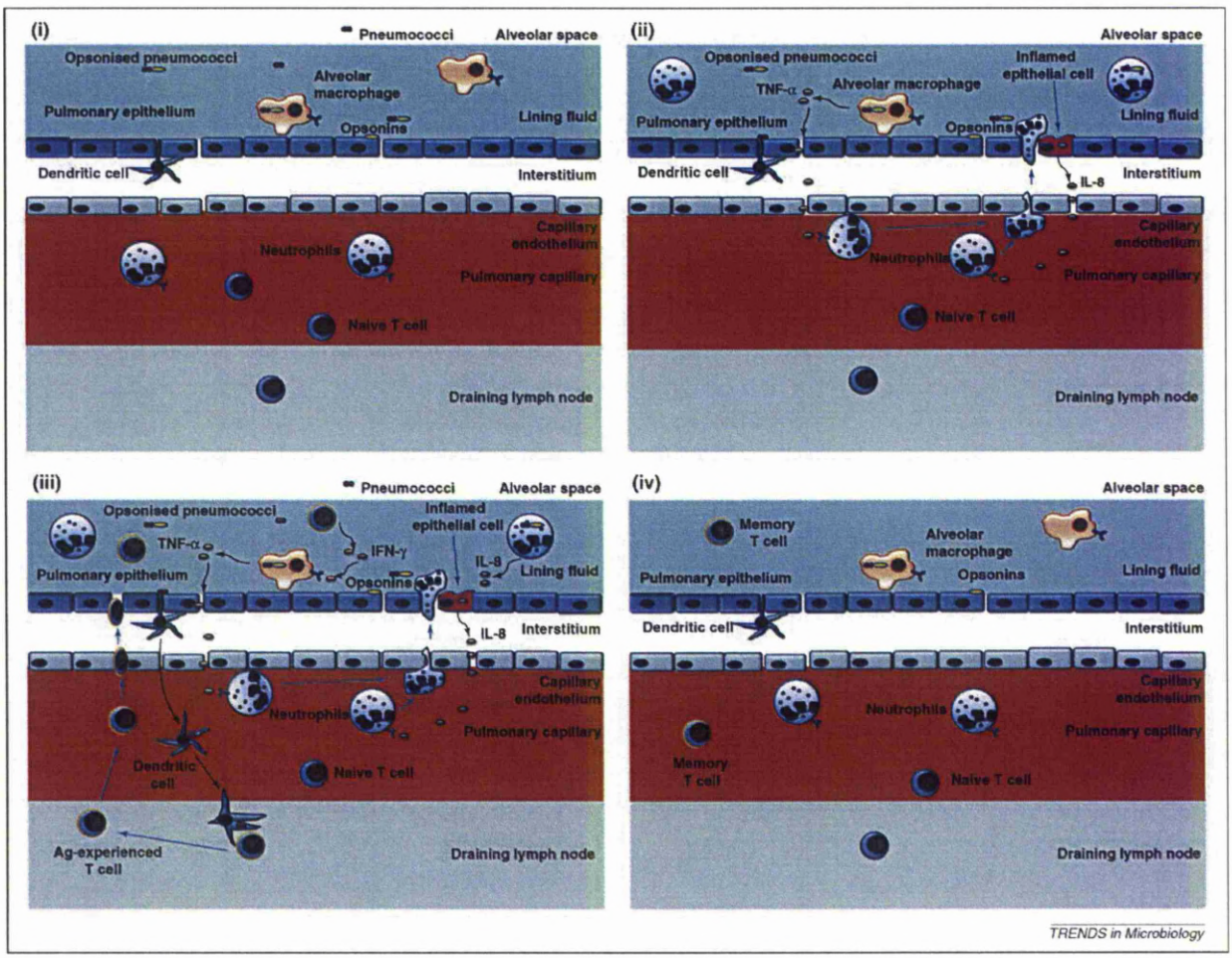

Figure 3. Pneumococcal clearance in the lung. Host defence in the lower respiratory tract is mediated by alveolar macrophages. **(i)** During early infection where the bacterial load is low, resident alveolar macrophages efficiently kill and phagocytise opsonised pneumococci in a quiescent manner, effectively preventing bacteria-dendritic cell interaction, and hence inhibiting initiation of T cell-mediated inflammatory responses. **(ii)** In situations where bacterial load exceeds the capability for macrophages to perform effective opsonophagocytosis, neutrophils are recruited following secretion of TNF-α by alveolar macrophages and/or IL-8 by epithelial cells. **(iii)** T cells are recruited following successful antigen presentation in the draining lymph nodes by pulmonary dendritic cells. These cells secrete IFN-γ which activates macrophages to kill internalised pneumococci and also promotes further TNF-α production by alveolar macrophages. **(iv)** Following clearance of pneumococci from the lungs, neutrophils, some macrophages and T cells undergo rapid apoptosis. Surviving T cells remain in the alveoli as resident effector memory cells.

apoptosis. Macrophage apoptosis leads to reduced TNF-α expression, which in turn results in reduced neutrophil recruitment and enhanced neutrophil apoptosis [46]. Dead cells are then cleared through phagocytosis, efferocytosis (clearance of apoptotic cells by phagocytes) and the normal function of the mucociliary escalator, whereas the surviving T cells remain in the alveoli as resident effector memory cells.

In summary, mucosal responses are critical in regulating pneumococcal carriage and defence against infection. Therefore, enhancing pulmonary mucosal immunity might be an effective strategy in the prevention of pneumococcal disease.

Evidence for vaccine-induced mucosal immunity against pneumococcus

Mucosal exposure to *S. pneumoniae* induces both mucosal and systemic humoral and cellular immune responses [25,26]. Enhancing these responses by mucosal vaccination is an attractive immunisation approach against pneumococcus as it mimics the natural route of infection. The success of other mucosal vaccines such as the trivalent oral poliovirus vaccine shows that this approach is a viable alternative of delivering vaccines [47]. There is evidence from mouse models, employing a similar route of vaccine delivery, showing that oral immunisation of PspA family fusion proteins delivered by attenuated *Salmonella enterica* serovar Typhimurium enhances protection against *S. pneumoniae* [48–51]. An important difference is that polio virus can replicate in the respiratory mucosa but pneumococcal antigens cannot. Nevertheless, there are several studies which have shown that mucosal immunisation can elicit protection against pneumococcal colonisation and infection (Table 1).

Protection against pneumococcal colonisation

Mucosal immunisation of experimental animals has been shown to elicit protection against carriage [29,52]. Intranasal immunisation of mice with killed, unencapsulated, whole cell pneumococci and cholera toxin adjuvant elicited protection against experimental colonisation. The protection was dependent on $CD4^+$ T cells and independent of antibody and bacterial serotype [29]. Furthermore, intranasal immunisation with a cholera toxin B subunit fused to the pneumococcal surface adhesin A (PsaA) also protected mice against colonisation with *S. pneumoniae* [52].

An ideal mucosal vaccine would include several pneumococcal proteins such as pneumolysin, PspA, PsaA or PspC [53]. Many of these proteins play a role in *S. pneumoniae* pathogenesis, and several are particularly relevant to protection against carriage [54]. Recently, Lu *et al.* demonstrated that a fusion conjugate, including cell wall polysaccharide coupled to pneumolysin and PsaA, delivered intranasally with cholera toxin, protected mice against experimental pneumococcal colonisation [31]. Mucosal vaccines based on protein combinations are more likely to exhibit coverage of all pneumococcal serotypes.

Protection against pneumococcal pneumonia

Pneumococcal conjugate vaccine is less effective against pneumonia than against invasive pneumococcal disease [7,8]. This observation might be as a result of difficulty either in the diagnosis of non-bacteraemic pneumococcal pneumonia or in distinguishing this diagnosis from other infective causes of pneumonia. Consequently, the chances of underreporting non-bacteraemic pneumococcal pneumonia as an endpoint in determining efficacy of the pneumococcal conjugate vaccine are high. By contrast, the intramuscular administration of the vaccine might result in a compartmentalisation of the immune response and a suboptimal lymphocyte trafficking to the pulmonary mucosa.

Mucosal vaccination has shown promising results in protection against pneumococcal lung infection [31,55–57]. Nasal administration of *Lactococcus lactis* increased the clearance rate of *S. pneumoniae* from the lung and prevented invasion of pneumococci into blood [57]. In these experiments, *L. lactis* increased phagocyte activation in

Table 1. Mouse models of mucosal immunisation with pneumococcal protein antigens[a]

Antigen or vaccine	Route	Immunogenicity	Correlates of protection	Protection against:	Refs
PspA	Intranasal	Mucosal and systemic	Antibodies in serum and saliva	Colonisation, pneumonia, sepsis	[60–62]
PspA/attenuated *Salmonella*	Oral	Mucosal and systemic	Antibodies in serum and vaginal fluids	Pneumonia, bacteraemia	[48–51,63]
PsaA	Oral	Mucosal and systemic	Antibodies in serum, BAL and intestinal fluid	Pneumonia, bacteraemia	[52,64,65]
PsaA/lactic acid bacteria	Intranasal	Mucosal and systemic	Antibodies in serum, saliva, nasal and bronchial washes	Colonisation	[66]
PotD	Intranasal	Mucosal and systemic	Antibodies in serum and saliva	Colonisation, pneumonia, bacteraemia	[67]
PsaA and PspA	Intranasal	Mucosal and systemic	Antibodies in serum and saliva	Colonisation	[68]
PspA and PspC	Intranasal	Mucosal and systemic	Antibodies in serum, vaginal washes, and BAL; cytokine responses in BAL, lung and splenic samples	Pneumonia, bacteraemia	[69,70]
PsaA, PdT and CWPS	Intranasal	Systemic	Antibodies in serum; T cell response in whole blood	Colonisation, pneumonia	[31,71]
GEM with PpmA, SlrA and IgA1p	Intranasal	Systemic	Antibodies in serum	Pneumonia	[72,73]
PCV	Intranasal	Mucosal and systemic	Antibodies in serum and nasal washes	Colonisation, otitis media	[74–77]

[a]Abbreviations: PspA, pneumococcal surface protein A; PsaA, 'pneumococcal surface adhesion A' protein; BAL, bronchoalveolar lavage; PotD, polyamine transport protein D; PspC, pneumococcal surface protein C; PdT, pneumolysin nontoxic derivative; CWPS, cell wall polysaccharide; GEM, Gram-positive enhancer matrix; PCV, pneumococcal conjugate vaccine.

lung, blood and bone marrow of the vaccinated mice [57]. Moreover, mucosal immunisation with caseinolytic protease, a conserved pneumococcal protein, induced the production of both systemic and mucosal antibodies and resulted in reduced lung bacterial load in a pneumococcal pneumonia model and prevented death in an intraperitoneal sepsis model [58]. Lastly, a mucosal vaccine made of recombinant PspA elicited protection against invasive pneumococcal challenge, characterised by increased secretion of IL-17 and IFN-γ by lung and spleen cells, respectively [55]. These data support the concept that mucosal immunisation might protect against both mucosal and systemic infection. However, the duration of protection afforded by these mucosal vaccines is not clear.

There are also some data to suggest that mucosally administered vaccines might actually provide better protection against both mucosal and systemic disease than conventional parenteral (systemic) vaccines. Immunisation of mice with lactococcal PspA vaccine elicited better protection against respiratory pneumococcal challenge than conventional parenteral PspA vaccine in intraperitoneal sepsis and intranasal respiratory infection models [56].

In summary, there are encouraging data to support the role of mucosal vaccination in protection against both mucosal and systemic pneumococcal disease. However, lack of a suitable adjuvant is a major obstacle to success, but cytokine adjuvants might be useful [59].

Concluding remarks and future directions

We have discussed the role of mucosal immunity and reviewed available data on mucosal immunisation against pneumococcal disease. There is evidence from murine studies to suggest that mucosal immunisation against pneumococci induces mucosal and systemic immunity more effectively than parenteral vaccination. The data from humans, however, are insufficient to draw firm conclusions. Further studies using lung, nasal and other mucosal samples from humans are needed.

Immediate priorities include the need to address the role of T cell-mediated immunity against pneumococcal colonisation and infection in humans. Such data might clarify the human correlates of protection or immunity to *S. pneumoniae*. These correlates of protection might then help in predicting efficacy to future vaccines. Research questions for future work in the field are outlined in Box 2.

Strategic decisions regarding future pneumococcal vaccines need to determine whether to focus on improving the current conjugate vaccines (by including more serotypes or replacing the carrier with a pneumococcal protein) or developing new pneumococcal protein based vaccines. Pneumococcal conjugate vaccines are currently being used in several developed countries because they provide good protection against systemic disease. Their main disadvantage is that they have limited serotype coverage and are less effective against mucosal disease. By contrast, pneumococcal protein based vaccines have the potential to offer universal coverage as well as offer protection against both mucosal and systemic disease if delivered through the mucosal route. Vaccination still remains the key to minimising the high burden of pneumococcal disease worldwide. We believe that alternative routes of immunisation (with conjugate vaccines or pneumococcal proteins) might help in improving the efficacy of pneumococcal vaccines to both mucosal and systemic disease.

Box 2. Questions for future research

- What are the correlates of protection (measurable signs of immunity) against carriage and disease as a result of *S. pneumoniae* in human mucosa?
- Which is the most effective mode of vaccine delivery to enhance both mucosal and systemic immunity to *S. pneumoniae*?
- What adjuvants are most effective for mucosal pneumococcal vaccine delivery?
- How does HIV infection affect mucosal immunity against *S. pneumoniae* in the respiratory tract?

Acknowledgements

This work received financial support from the Wellcome Trust of Great Britain and the Commonwealth Scholarship Commission, and the National Institute for Health Research Biomedical Research Centre in Microbial Disease (Liverpool). It forms part of the Malawi-Liverpool-Wellcome Trust Clinical Research Programme.

References

1 Kadioglu, A. *et al.* (2008) The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat. Rev. Microbiol.* 6, 288–301
2 Bogaert, D. *et al.* (2004) *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect. Dis.* 4, 144–154
3 Jover, F. *et al.* (2008) A comparative study of bacteremic and non-bacteremic pneumococcal pneumonia. *Eur. J. Intern. Med.* 19, 15–21
4 Wardlaw, T. *et al.* (2006) *Pneumonia: The Forgotten Killer of Children*, UNICEF/WHO
5 Turner, D. (2008) *Improving Global Health by Preventing Pneumococcal Disease*, APPG/UK Government
6 King, J.C., Jr *et al.* (1996) Comparison of the safety and immunogenicity of a pneumococcal conjugate with a licensed polysaccharide vaccine in human immunodeficiency virus and non-human immunodeficiency virus-infected children. *Pediatr. Infect. Dis. J.* 15, 192–196
7 Cutts, F.T. *et al.* (2005) Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. *Lancet* 365, 1139–1146
8 Qazi, S. *et al.* (2007) *Global Action Plan for the Prevention and Control of Pneumonia (GAPP): Report of an Informal Consultation*, UNICEF/WHO
9 Cheung, Y.B. *et al.* (2009) Nasopharyngeal carriage of *Streptococcus pneumoniae* in Gambian children who participated in a 9-valent pneumococcal conjugate vaccine trial and in their younger siblings. *Pediatr. Infect. Dis. J.* 28, 990–995
10 Du Clos, T.W. and Mold, C. (2004) C-reactive protein: an activator of innate immunity and a modulator of adaptive immunity. *Immunol. Res.* 30, 261–277
11 Gould, J.M. and Weiser, J.N. (2001) Expression of C-reactive protein in the human respiratory tract. *Infect. Immun.* 69, 1747–1754
12 Gould, J.M. and Weiser, J.N. (2002) The inhibitory effect of C-reactive protein on bacterial phosphorylcholine platelet-activating factor receptor-mediated adherence is blocked by surfactant. *J. Infect. Dis.* 186, 361–371
13 Attali, C. *et al.* (2008) The interaction of *Streptococcus pneumoniae* with plasmin mediates transmigration across endothelial and epithelial monolayers by intercellular junction cleavage. *Infect. Immun.* 76, 5350–5356
14 Roy, S. *et al.* (2002) MBL genotype and risk of invasive pneumococcal disease: a case-control study. *Lancet* 359, 1569–1573
15 Brown, J.S. *et al.* (2002) The classical pathway is the dominant complement pathway required for innate immunity to *Streptococcus*

pneumoniae infection in mice. *Proc. Natl. Acad. Sci. U. S. A.* 99, 16969–16974

16 Lysenko, E.S. *et al.* (2005) The role of innate immune responses in the outcome of interspecies competition for colonization of mucosal surfaces. *PLoS Pathog.* 1, e1

17 Kerr, A.R. *et al.* (2005) Innate immune defense against pneumococcal pneumonia requires pulmonary complement component C3. *Infect. Immun.* 73, 4245–4252

18 van Rossum, A.M. *et al.* (2005) Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. *Infect. Immun.* 73, 7718–7726

19 Nelson, A.L. *et al.* (2007) Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infect. Immun.* 75, 83–90

20 Martner, A. *et al.* (2009) *Streptococcus pneumoniae* autolysis prevents phagocytosis and production of phagocyte-activating cytokines. *Infect. Immun.* 77, 3826–3837

21 Matthias, K.A. *et al.* (2008) Neutrophil-toxin interactions promote antigen delivery and mucosal clearance of *Streptococcus pneumoniae*. *J. Immunol.* 180, 6246–6254

22 O'Brien, K.D. *et al.* (2008) *Nasopharyngeal Carriage*, ASM Press

23 Weiser, J.N. *et al.* (2003) Antibody-enhanced pneumococcal adherence requires IgA1 protease. *Proc. Natl. Acad. Sci. U. S. A.* 100, 4215–4220

24 Weinberger, D.M. *et al.* (2008) Epidemiologic evidence for serotype-specific acquired immunity to pneumococcal carriage. *J. Infect. Dis.* 197, 1511–1518

25 Goldblatt, D. *et al.* (2005) Antibody responses to nasopharyngeal carriage of *Streptococcus pneumoniae* in adults: a longitudinal household study. *J. Infect. Dis.* 192, 387–393

26 Zhang, Q. *et al.* (2006) Serum and mucosal antibody responses to pneumococcal protein antigens in children: relationships with carriage status. *Eur. J. Immunol.* 36, 46–57

27 Gordon, S.B. *et al.* (2000) Intracellular trafficking and killing of *Streptococcus pneumoniae* by human alveolar macrophages are influenced by opsonins. *Infect. Immun.* 68, 2286–2293

28 McCool, T.L. and Weiser, J.N. (2004) Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. *Infect. Immun.* 72, 5807–5813

29 Malley, R. *et al.* (2005) $CD4^+$ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proc. Natl. Acad. Sci. U. S. A.* 102, 4848–4853

30 Bogaert, D. *et al.* (2009) Impaired innate and adaptive immunity to *Streptococcus pneumoniae* and its effect on colonization in an infant mouse model. *Infect. Immun.* 77, 1613–1622

31 Lu, Y.J. *et al.* (2009) Protection against pneumococcal colonization and fatal pneumonia by a trivalent conjugate of a fusion protein with the cell wall polysaccharide. *Infect. Immun.* 77, 2076–2083

32 Lu, Y.J. *et al.* (2008) Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog.* 4, e1000159

33 Malley, R. *et al.* (2006) Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. *Infect. Immun.* 74, 2187–2195

34 Zhang, Z. *et al.* (2009) Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J. Clin. Invest.* 119, 1899–1909

35 Gordon, S.B. and Read, R.C. (2002) Macrophage defences against respiratory tract infections. *Br. Med. Bull.* 61, 45–61

36 Stockley, R. (1995) Humoral and cellular mechanisms, In *Respiratory Medicine* (2nd edn) (Brewis, R.C. *et al.*, eds), WB Saunders

37 Riches, D.W.H. and Fenton, M.J. (2009) Monocytes, macrophages and dendritic cells of the lung. In *Murray and Nadel's Textbook of Respiratory Medicine* (Online version) (Mason, R.B., *et al.*, eds), Elsevier

38 Marriott, H.M. and Dockrell, D.H. (2007) The role of the macrophage in lung disease mediated by bacteria. *Exp. Lung Res.* 33, 493–505

39 Blumenthal, R.L. *et al.* (2001) Human alveolar macrophages induce functional inactivation in antigen-specific CD4 T cells. *J. Allergy Clin. Immunol.* 107, 258–264

40 Kirby, A.C. *et al.* (2005) The role played by tumor necrosis factor during localized and systemic infection with *Streptococcus pneumoniae*. *J. Infect. Dis.* 191, 1538–1547

41 Murdoch, C. *et al.* (1999) Functional expression of chemokine receptor CXCR4 on human epithelial cells. *Immunology* 98, 36–41

42 Zarbock, A. and Ley, K. (2008) Mechanisms and consequences of neutrophil interaction with the endothelium. *Am. J. Pathol.* 172, 1–7

43 Hirst, R.A. *et al.* (2004) The role of pneumolysin in pneumococcal pneumonia and meningitis. *Clin. Exp. Immunol.* 138, 195–201

44 Kadioglu, A. *et al.* (2000) Host cellular immune response to pneumococcal lung infection in mice. *Infect. Immun.* 68, 492–501

45 Kirby, A.C. *et al.* (2007) Pulmonary dendritic cells and alveolar macrophages are regulated by gammadelta T cells during the resolution of *S. pneumoniae*-induced inflammation. *J. Pathol.* 212, 29–37

46 Marriott, H.M. *et al.* (2006) Decreased alveolar macrophage apoptosis is associated with increased pulmonary inflammation in a murine model of pneumococcal pneumonia. *J. Immunol.* 177, 6480–6488

47 Onorato, I.M. *et al.* (1991) Mucosal immunity induced by enhance-potency inactivated and oral polio vaccines. *J. Infect. Dis.* 163, 1–6

48 Kang, H.Y. *et al.* (2002) Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated *Salmonella enterica* serovar Typhimurium vaccine. *Infect. Immun.* 70, 1739–1749

49 Li, Y. *et al.* (2009) Evaluation of new generation *Salmonella enterica* serovar Typhimurium vaccines with regulated delayed attenuation to induce immune responses against PspA. *Proc. Natl. Acad. Sci. U. S. A.* 106, 593–598

50 Nayak, A.R. *et al.* (1998) A live recombinant avirulent oral *Salmonella* vaccine expressing pneumococcal surface protein A induces protective responses against *Streptococcus pneumoniae*. *Infect. Immun.* 66, 3744–3751

51 Xin, W. *et al.* (2009) PspA family fusion proteins delivered by attenuated *Salmonella enterica* serovar Typhimurium extend and enhance protection against *Streptococcus pneumoniae*. *Infect. Immun.* 77, 4518–4528

52 Pimenta, F.C. *et al.* (2006) Intranasal immunization with the cholera toxin B subunit-pneumococcal surface antigen A fusion protein induces protection against colonization with *Streptococcus pneumoniae* and has negligible impact on the nasopharyngeal and oral microbiota of mice. *Infect. Immun.* 74, 4939–4944

53 Ogunniyi, A.D. *et al.* (2007) Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. *Infect. Immun.* 75, 350–357

54 Ogunniyi, A.D. *et al.* (2007) Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of *Streptococcus pneumoniae* D39 in a mouse model. *Infect. Immun.* 75, 1843–1851

55 Ferreira, D.M. *et al.* (2009) Characterization of protective mucosal and systemic immune responses elicited by pneumococcal surface protein PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice. *Clin. Vaccine Immunol.* 16, 636–645

56 Hanniffy, S.B. *et al.* (2007) Mucosal delivery of a pneumococcal vaccine using *Lactococcus lactis* affords protection against respiratory infection. *J. Infect. Dis.* 195, 185–193

57 Medina, M. *et al.* (2008) Nasal administration of *Lactococcus lactis* improves local and systemic immune responses against *Streptococcus pneumoniae*. *Microbiol. Immunol.* 52, 399–409

58 Cao, J. *et al.* (2008) Mucosal immunization with purified ClpP could elicit protective efficacy against pneumococcal pneumonia and sepsis in mice. *Microbes Infect.* 10, 1536–1542

59 Wright, A.K. *et al.* (2008) Prospects for use of interleukin-12 as a mucosal adjuvant for vaccination of humans to protect against respiratory pneumococcal infection. *Vaccine* 26, 4893–4903

60 Wu, H.Y. *et al.* (1997) Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. *J. Infect. Dis.* 175, 839–846

61 Campos, I.B. *et al.* (2008) Nasal immunization of mice with *Lactobacillus casei* expressing the Pneumococcal Surface Protein A: induction of antibodies, complement deposition and partial protection against *Streptococcus pneumoniae* challenge. *Microbes Infect.* 10, 481–488

62 Yamamoto, M. *et al.* (1997) Oral immunization with PspA elicits protective humoral immunity against *Streptococcus pneumoniae* infection. *Infect. Immun.* 65, 640–644

63 Park, S.M. *et al.* (2008) MyD88 signaling is not essential for induction of antigen-specific B cell responses but is indispensable for protection against *Streptococcus pneumoniae* infection following oral vaccination with attenuated *Salmonella* expressing PspA antigen. *J. Immunol.* 181, 6447–6455
64 Seo, J.Y. *et al.* (2002) Cross-protective immunity of mice induced by oral immunization with pneumococcal surface adhesin a encapsulated in microspheres. *Infect. Immun.* 70, 1143–1149
65 Areas, A.P. *et al.* (2004) Expression and characterization of cholera toxin B-pneumococcal surface adhesin A fusion protein in *Escherichia coli*: ability of CTB-PsaA to induce humoral immune response in mice. *Biochem. Biophys. Res. Commun.* 321, 192–196
66 Oliveira, M.L. *et al.* (2006) Induction of systemic and mucosal immune response and decrease in *Streptococcus pneumoniae* colonization by nasal inoculation of mice with recombinant lactic acid bacteria expressing pneumococcal surface antigen A. *Microbes Infect.* 8, 1016–1024
67 Shah, P. *et al.* (2009) Mucosal immunization with polyamine transport protein D (PotD) protects mice against nasopharyngeal colonization with *Streptococcus pneumoniae*. *Exp. Biol. Med. (Maywood)* 234, 403–409
68 Briles, D.E. *et al.* (2000) Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. *Infect. Immun.* 68, 796–800
69 Ferreira, D.M. *et al.* (2009) Characterization of protective mucosal and systemic immune responses elicited by Pneumococcal Surface Proteins PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice. *Clin. Vaccine Immunol.* 16, 636–645
70 Xin, W. *et al.* (2008) Analysis of type II secretion of recombinant pneumococcal PspA and PspC in a *Salmonella enterica* serovar Typhimurium vaccine with regulated delayed antigen synthesis. *Infect. Immun.* 76, 3241–3254
71 Arevalo, M.T. *et al.* (2009) Mucosal vaccination with a multicomponent adenovirus-vectored vaccine protects against *Streptococcus pneumoniae* infection in the lung. *FEMS Immunol. Med. Microbiol.* 55, 346–351
72 Audouy, S.A. *et al.* (2006) Lactococcus lactis GEM particles displaying pneumococcal antigens induce local and systemic immune responses following intranasal immunization. *Vaccine* 24, 5434–5441
73 Audouy, S.A. *et al.* (2007) Development of lactococcal GEM-based pneumococcal vaccines. *Vaccine* 25, 2497–2506
74 Sabirov, A. and Metzger, D.W. (2008) Intranasal vaccination of infant mice induces protective immunity in the absence of nasal-associated lymphoid tissue. *Vaccine* 26, 1566–1576
75 Sabirov, A. and Metzger, D.W. (2006) Intranasal vaccination of neonatal mice with polysaccharide conjugate vaccine for protection against pneumococcal otitis media. *Vaccine* 24, 5584–5592
76 Lynch, J.M. *et al.* (2003) Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. *Infect. Immun.* 71, 4780–4788
77 Jakobsen, H. *et al.* (1999) Intranasal immunization with pneumococcal polysaccharide conjugate vaccines protects mice against invasive pneumococcal infections. *Infect. Immun.* 67, 4128–4133
78 Kadioglu, A. *et al.* (2002) Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. *Infect. Immun.* 70, 2886–2890
79 Orihuela, C.J. *et al.* (2004) Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *J. Infect. Dis.* 190, 1661–1669
80 Berry, A.M. *et al.* (1999) Comparative virulence of *Streptococcus pneumoniae* strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. *Infect. Immun.* 67, 981–985
81 Benton, K.A. *et al.* (1995) A pneumolysin-negative mutant of *Streptococcus pneumoniae* causes chronic bacteremia rather than acute sepsis in mice. *Infect. Immun.* 63, 448–455

Bronchoalveolar CD4$^+$ T cell responses to respiratory antigens are impaired in HIV-infected adults

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Received 19 October 2010
Accepted 27 January 2011
Published Online First 25 February 2011

ABSTRACT

Rationale HIV-infected adults are at an increased risk of lower respiratory tract infections. HIV infection impairs systemic acquired immunity, but there is limited information in humans on HIV-related cell-mediated immune defects in the lung.

Objective To investigate antigen-specific CD4$^+$ T cell responses to influenza virus, *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* antigens in bronchoalveolar lavage (BAL) and peripheral blood between HIV-infected individuals and HIV-uninfected Malawian adults.

Methods We obtained BAL fluid and blood from HIV-infected individuals (n=21) and HIV-uninfected adults (n=24). We determined the proportion of T cell subsets including naive, memory and regulatory T cells using flow cytometry, and used intracellular cytokine staining to identify CD4$^+$ T cells recognising influenza virus-, *S pneumoniae*- and *M tuberculosis*-antigens.

Main results CD4$^+$ T cells in BAL were predominantly of effector memory phenotype compared to blood, irrespective of HIV status ($p<0.001$). There was immune compartmentalisation with a higher frequency of antigen-specific CD4$^+$ T cells against influenza virus, *S pneumoniae* and *M tuberculosis* retained in BAL compared to blood in HIV-uninfected adults ($p<0.001$ in each case). Influenza virus- and *M tuberculosis*-specific CD4$^+$ T cell responses in BAL were impaired in HIV-infected individuals: proportions of total antigen-specific CD4$^+$ T cells and of polyfunctional IFN-γ and TNF-α-secreting cells were lower in HIV-infected individuals than in HIV-uninfected adults ($p<0.05$ in each case).

Conclusions BAL antigen-specific CD4$^+$ T cell responses against important viral and bacterial respiratory pathogens are impaired in HIV-infected adults. This might contribute to the susceptibility of HIV-infected adults to lower respiratory tract infections such as pneumonia and tuberculosis.

INTRODUCTION

Respiratory infections are a leading cause of death in lower income countries, accounting for approximately three million deaths a year.[1] Infants, the elderly and immunocompromised individuals are particularly susceptible to these infections. In particular, HIV-infected individuals are 30 times more likely than uninfected adults to suffer from bacterial pneumonia or active tuberculosis.[2 3] Southern Africa is the region with the world's highest burden of HIV infection, with over nine countries, including Malawi, having an estimated HIV prevalence that is greater than 10%.[4]

Defence against respiratory infection involves mucosal and systemic immunity.[5] Antigen-specific CD4$^+$ T cells are important as they protect against respiratory infections.[6–8] Cytokines secreted by CD4$^+$ T cells, such as IFN-γ, TNF-α, IL-2, IL-17 and IL-22,[9–12] are critical to the activation of macrophages[9] and the recruitment of neutrophils,[10] and enhance the magnitude and quality of CD8$^+$ T cell responses.[12] Immune protection against common viral and bacterial respiratory pathogens depends on the integrity of these effector responses. There is limited information about the phenotype and function of these CD4$^+$ T cells within the human lung.

Studies of the human lung suggest that mechanisms of local intrapulmonary immunity may differ from those mediating systemic immunity. Influenza virus antigen-specific memory CD4$^+$ T cells from lung tissue were present at higher frequencies and produced more IFN-γ than those from peripheral blood in patients undergoing lobectomy for a localised solitary peripheral lung carcinoma who had no symptoms of upper respiratory infection.[13] In patients with tuberculosis, IFN-γ and TNF-α responses to Purified Protein Derivative (PPD) were stronger by CD4$^+$ T cells from BAL fluid than by CD4$^+$ T cells from peripheral blood.[14]

The decline in immunity caused by HIV is not equally distributed among immunological sites. In particular, the depletion of CD4$^+$ T cells primarily occurs at sites of 'persistent inflammation' such as the mucosa, which may leave individuals vulnerable to acute infections. Brenchley *et al* showed that there was a rapid depletion of mucosal gut T cells during early HIV infection while pulmonary CD4$^+$ T cell depletion was less acute.[15] Clinical evidence indicates that there is a high burden of pneumococcal pneumonia and tuberculosis early on in HIV infection when peripheral CD4$^+$ T cell counts are relatively stable.[16 17] We hypothesised that HIV infection preferentially depletes antigen-specific T cells against common respiratory pathogens within the lung compartment, which predisposes individuals to respiratory infections.

The authors have compared baseline T cell phenotypes and antigen-specific CD4$^+$ T cells in BAL and peripheral blood, between HIV-infected individuals and HIV-uninfected adults. The aim of the authors was to compare T cell phenotypes in BAL and peripheral blood between the two groups of subjects; to assess antigen-specific CD4$^+$ T cell responses to common respiratory antigens; and to investigate whether HIV infection differentially affects the lung and peripheral blood compartments.

METHODS

Participants

Adult volunteers with no recent history of severe respiratory diseases and a normal chest x-ray were recruited by advertisement in the Queen Elizabeth Central Hospital, Blantyre, Malawi. All participants gave written-informed-consent to HIV testing, venesection and bronchoscopy. The authors enrolled HIV-uninfected adults and asymptomatic anti-retroviral therapy naive HIV-infected individuals (WHO stage 1) into the study. The exclusion criteria for the study were as follows: the presence of other immunocompromising illnesses such as diabetes and cancer, the use of immunosuppressive drugs, cigarette smoking, moderate or severe anaemia (HB<8 g/dl), and known or possible pregnancy. This study complies with local institutional guidelines and was approved by the College of Medicine Research Ethics Committee of the University of Malawi (COMREC P.01/09/717) and the Liverpool School of Tropical Medicine Research Ethics Committee (LSTM REC 08.61).

Sample collection and processing

Peripheral blood samples were collected on all subjects. Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density centrifugation using Lymphoprep (Axis-shield, Norway) according to the manufacturer's instructions. Bronchoscopy and BAL collection was carried out as previously described.[18] The BAL samples were filtered and spun to obtain a cell pellet. The cells were counted and re-suspended in complete cell culture media (containing RPMI, L-glutamine, penicillin/streptomycin and HEPES (all from Sigma-Aldrich, UK) with 2% (vol/vol) heat-inactivated human AB serum (National Blood Services, Blantyre)).

Phenotyping of T cell subsets

PBMCs and BAL cells were stained with fluorochrome conjugate antibodies when cell numbers were sufficient. Anti-CD3 fluorescein isothiocyanate (FITC), anti-CD4 Pacific blue, anti-CD8 allophycocyanin-H7 (APC-H7), anti-CD45RA phycoerythrin (PE), and anti-CCR7 allophycocyanin (APC) (all antibodies from BD Bioscience, UK) were used to characterise: naive ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), effector memory ($CD45RA^-CCR7^-$) and terminal effector T cells ($CD45RA^+CCR7^-$).[19] Anti-CD4 pacific blue, anti-CD25 FITC (all antibodies from BD Bioscience, UK) and anti-FoxP3 PE (eBioscience, UK) were used to characterise regulatory T cells ($CD4^+CD25^{hi}FoxP3^+$).[20] The samples were acquired on CyAn ADP 9 Colour flow cytometer (Beckman Coulter, USA) and analysed using FlowJo (TreeStar, USA).

Intracellular cytokine staining

PBMCs and BAL cells re-suspended in complete cell culture media were cultured in a volume of 200 µl in a 96 well plate and stimulated with influenza vaccine (0.45 µg/ml), pneumococcal cell culture supernatant (8 µg/ml) or Purified Protein Derivative (PPD, 10 µg/ml) for 2 h. Brefeldin A (1 µl) (BD Bioscience, UK) was added at 2 h and the cells were cultured for a further 16 h. Cells were harvested and stained with Violet Viability dye (LIVE/DEAD® Fixable Dead Cell Stain kit, Invitrogen, UK) as per manufacturer's instructions. Cells were then surface stained with anti-CD4 FITC and CD8 PerCP (all BD Bioscience, UK). Next, cells were permeabilised and fixed using Cytofix/Cytoperm (BD Bioscience, UK) as per manufacturer's instructions. The cells were then stained with anti-interferon-gamma (IFN-γ) APC and anti-tumour necrosis factor-alpha (TNF-α) Alexa flour 488 antibodies (all BD Bioscience, UK) to detect intracellular cytokines. Lastly, cells were washed with 1x Perm Wash (BD Bioscience, UK), re-suspended in FACS flow and acquired on a flow on CyAn ADP 9 Colour flow cytometer (Beckman Coulter, USA). Flow cytometry analysis was done using FlowJo (TreeStar, USA).

Statistical analysis

Statistical analyses and graphical presentation were carried out using Graphpad Prism 5 (GraphPad Software, USA). Student t tests were used for the volunteer demographic data with the exception of gender, where a χ^2 test was used instead. For the experimental data, Mann–Whitney U test was used for non-paired data and Wilcoxon sign ranked test for paired data. Results are given as mean with ranges or medians with IQRs. Differences were considered statistically significant if p values were less than 0.05.

RESULTS

Demographic characteristics of study population

Basic demographics are shown in table 1. HIV-uninfected Malawian adults ((n=24, females 11) mean age 38 years)) and asymptomatic HIV-infected adults ((n=21, females 11) mean age 40 years)) participated in the study. The mean CD4 count for HIV-infected individuals was 375 cells/µl. All participants were asymptomatic and had no recent history of respiratory infection or tuberculosis. The mean BAL cell concentration was comparable between HIV-infected individuals and HIV-uninfected adults (16.2×10^6cells/100 ml vs 20.5×10^6cells/100 ml respectively; p=0.1134), but the proportion of lymphocytes in the BAL cells was higher in HIV-infected individuals than HIV-uninfected adults (17.8% vs 9.0%; p=0.0106).

Proportions of naive, memory and regulatory T cells in BAL and peripheral blood

CD4 and CD8 T cells

There was a lower proportion of $CD4^+$ T cells in the total $CD3^+$ T cell population in HIV-infected individuals compared to HIV-uninfected adults in both BAL (median 35.0% vs 65.5%, p<0.001) and peripheral blood (median 38.5% vs 61.2%, p<0.001). The proportion of $CD8^+$ T cells in the total $CD3^+$ T cell population was higher in HIV-infected individuals compared to HIV-uninfected adults in both BAL (median 59.7% vs 34.5%, p<0.05) and peripheral blood (median 61.5% vs 38.8%, p<0.05) (figure 1A).

Naive and memory T cells

To examine whether the proportion of T cell subsets was similar between compartments and whether they were altered by HIV infection, the authors measured expression of CD45RA

Table 1 Characteristics of subjects enrolled in the study

	HIV-uninfected adults n=24	HIV-infected adults n=21	p Value
Sex: female/male	11/13	11/10	0.6611
Age, mean yr±SD	38±17	40±18	0.7733
Systemic mean $CD4^+$ cells/µl±SD	767±208	375±172	<0.0001
BAL cells/100ml BALF, mean±SD	$16.2\times10^6\pm8.2\times10^6$	$20.5\times10^6\pm9.5\times10^6$	0.1134
BAL lymphocyte %, mean±SD	9.0±7.3	17.8±14.1	0.0106
WHO Stage	NA	Stage 1	

BAL, bronchoalveolar lavage; BALF, BAL fluid; N/A, not applicable.

Thorax 2011;**66**:375–382. doi:10.1136/thx.2010.153825

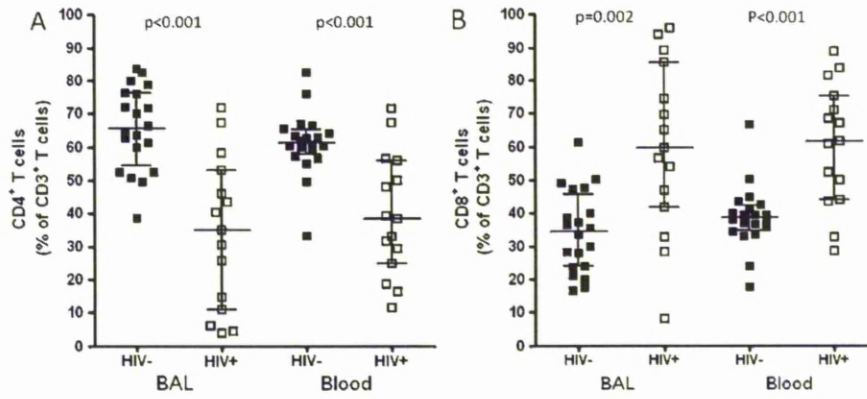

Figure 1 Lower proportion of $CD4^+$ T cells in BAL and peripheral blood of HIV-infected individuals compared to HIV-uninfected adults. T lymphocytes obtained from BAL and peripheral blood were stained with anti-CD3 FITC, anti-CD4 Pacific blue and anti-CD8 APC-H7 fluorochrome conjugated antibodies. (A) The data shows a lower proportion of $CD4^+$ T cells in BAL and peripheral blood of HIV-infected individuals compared to HIV-uninfected adults. (B) The data shows a higher proportion of $CD8^+$ T cells in BAL and peripheral blood of HIV-infected individuals compared to HIV-uninfected adults. Significance was assessed using Mann–Whitney U test. Black horizontal bars represent median and IQRs.

and CCR7 on $CD4^+$ and $CD8^+$ T cells—as described in the materials and methods section. BAL $CD4^+$ and $CD8^+$ T cells were predominantly of effector memory phenotype ($CD4^+CD45RA^-CCR7^-$, median 98%; $CD8^+CD45RA^-CCR7^-$, median 90%) compared to peripheral blood $CD4^+$ and $CD8^+$ T cells which were distributed among naive ($CD4^+CD45RA^+CCR7^+$, median 30%), central memory ($CD4^+CD45RA^-CCR7^+$, median 37%; $CD8^+CD45RA^-CCR7^+$, median 5%), effector memory ($CD4^+$ $CD45RA^-CCR7^-$, median 37%; $CD8^+$ $CD45RA^-CCR7^-$, median 20%) and terminal effector phenotypes ($CD4^+CD45RA^+CCR7^-$, median 6%; $CD8^+$ $CD45RA^+CCR7^-$, median 45%) (figure 2). In BAL, there was a higher proportion of effector memory cells in HIV-infected individuals compared to HIV-uninfected adults (CD4, median 98.9% vs 98.1% p=0.03; CD8, median 96.3% vs 90.3% p=0.03) and a lower proportion of central memory T cells (CD4, median 0.37% vs 1.42% p=0.003; CD8, median 0.44% vs 1.88% p=0.007) (figure 2A,B). In peripheral blood, there was no difference in peripheral blood $CD4^+$ T cell subsets between HIV-infected and HIV-uninfected groups. However, the HIV-infected group had a higher proportion of $CD8^+$ effector memory T cells (median 29.6% vs 17.8%, p=0.007) and a lower proportion of $CD8^+$ terminal effector T cells (median 22.2% vs 45.0%, p=0.01) than the HIV-uninfected group (figure 2C,D).

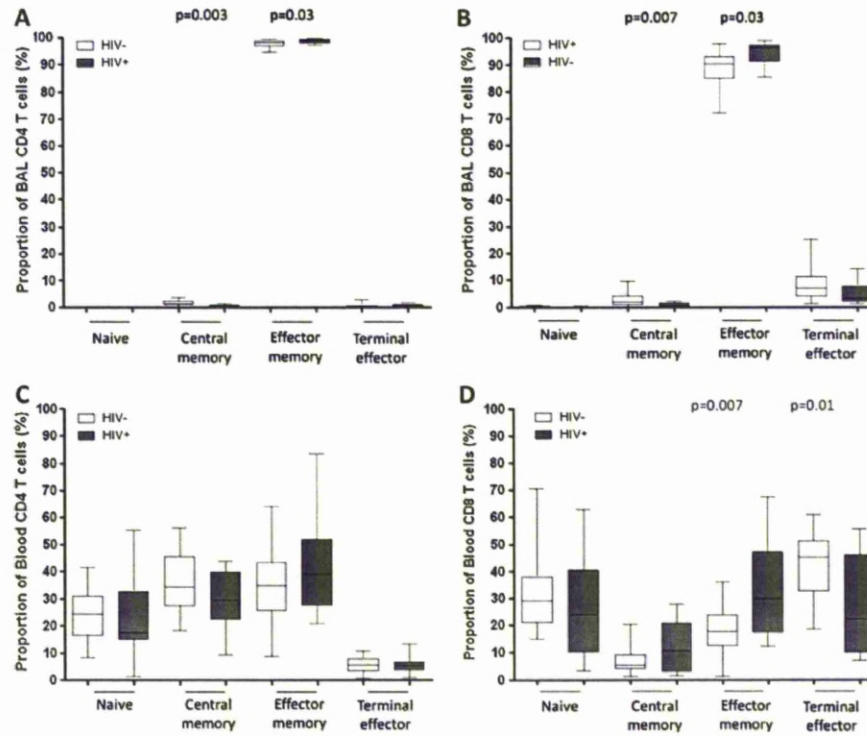

Figure 2 The proportions of naive and memory T cell subsets are different between BAL and peripheral blood, and are altered during HIV infection. T lymphocytes obtained from BAL and peripheral blood were stained with anti-CD3 FITC, anti-CD4 Pacific blue, anti-CD8 APC-H7, anti-CD45RA PE and anti-CCR7 APC fluorochrome conjugated antibodies. The proportion of naïve ($CD45RA^+CCR7^+$), central memory ($CD45RA^-CCR7^+$), effector memory ($CD45RA^-CCR7^-$) and terminal effector ($CD45RA^+CCR7^-$) were defined. (A, B, C, D) The data shows that BAL T cells (upper) were predominantly of effector memory phenotype compared to peripheral blood (lower), in which T cells were distributed among naive, central memory, effector memory and terminal effector phenotypes. (A, B) The data shows a higher proportion of effector memory and lower proportion of central memory BAL $CD4^+$ (left) and $CD8^+$ (right) T cells in HIV-infected individuals compared to HIV-uninfected adults. (C, D) The data shows no difference in peripheral blood $CD4^+$ T cell subsets between HIV-infected individuals compared to HIV-uninfected adults (left), but there was a higher proportion of $CD8^+$ effector memory and a lower proportion of $CD8^+$ terminal effector in HIV-infected individuals compared to HIV-uninfected adults (right). Black horizontal bars represent median and IQRs. Statistical significance was analysed by the Mann–Whitney U test. p value <0.05 was used to determine statistical significance.

Regulatory T cells (Tregs)

We investigated the hypothesis that in HIV-infected individuals, persistent immune activation by HIV results in a higher frequency of regulatory T cells. Blood and BAL Tregs were defined as $CD4^+CD25^{hi}FoxP3^+$ as described in the materials and methods (figure 3A). The proportions of Tregs in HIV-infected individuals were similar in BAL and peripheral blood (median 3.7% vs 3%, p>0.01), but were higher in BAL compared to peripheral blood in HIV-uninfected adults (median 4.3% vs 1.5%, p<0.001) (figure 3B). There was a higher proportion of Tregs in the peripheral blood of HIV-infected individuals than in the HIV-uninfected adults (median 3% vs 1.5%, p<0.001). However, in BAL the proportions were similar between the groups (median 3.7% vs 4.3%, p>0.01) (figure 3B). Absolute counts, however, of Tregs in peripheral blood were similar between HIV-uninfected adults and HIV-infected individuals (median 11 cells/μl vs 9 cells/μl, p>0.01) (figure 3C).

Antigen-specific $CD4^+$ T cells against influenza virus, *S pneumoniae* and *M tuberculosis* in BAL and peripheral blood

To investigate respiratory pathogen antigen-specific T cell responses in BAL and peripheral blood, we measured the quality and magnitude of the $CD4^+$ T cell response to influenza virus, *S pneumoniae* and *M tuberculosis* antigens using intracellular cytokine staining. We detected antigen-specific $CD4^+$ T cells against influenza virus, *S pneumoniae* and *M tuberculosis* in BAL and peripheral blood (figure 4).

Differences between BAL and peripheral blood compartments

There was a higher percentage frequency of antigen-specific $CD4^+$ T cells against influenza virus (median 0.51% vs 0.13%, p<0.001), *S pneumoniae* (median 0.59% vs 0.11%, p<0.001) and *M tuberculosis* (median 5.53% vs 0.20%, p<0.001) in BAL compared to peripheral blood (figure 5A–C). The proportion of antigen-specific $CD4^+$ T cells against influenza virus, *S pneumoniae* and *M tuberculosis* producing either IFN-γ alone, TNF-α alone or both cytokines were different between BAL and peripheral blood (figure 6A–C). Background responses were subtracted from all the antigen-specific $CD4^+$ T cell responses.

Differences between HIV-infected and HIV-uninfected adults

The percentage frequency of antigen-specific $CD4^+$ T cells against influenza virus (median 0.15% vs 0.51%, p<0.05) and *M tuberculosis* (median 0.5% vs 5.53%, p<0.05) antigens were lower in HIV-infected individuals compared to HIV-uninfected adults in BAL (figure 5A,C). However, this was not the case in peripheral blood where the percentage frequencies were comparable (Influenza virus, median 0.3% vs 0.13%, p>0.05; *M tuberculosis*, median 0.12% vs 0.19% p>0.05)(figure 5A,C). The percentage frequency of antigen-specific $CD4^+$ T cells against *S pneumoniae* were similar in HIV-infected compared to HIV-uninfected adults in BAL (median 0.84% vs 0.59%, p>0.05). However, in peripheral blood the percentage frequency was higher in HIV-infected compared to HIV-uninfected adults (median 0.2% vs 0.1%, p=0.02) (figure 5B). Background

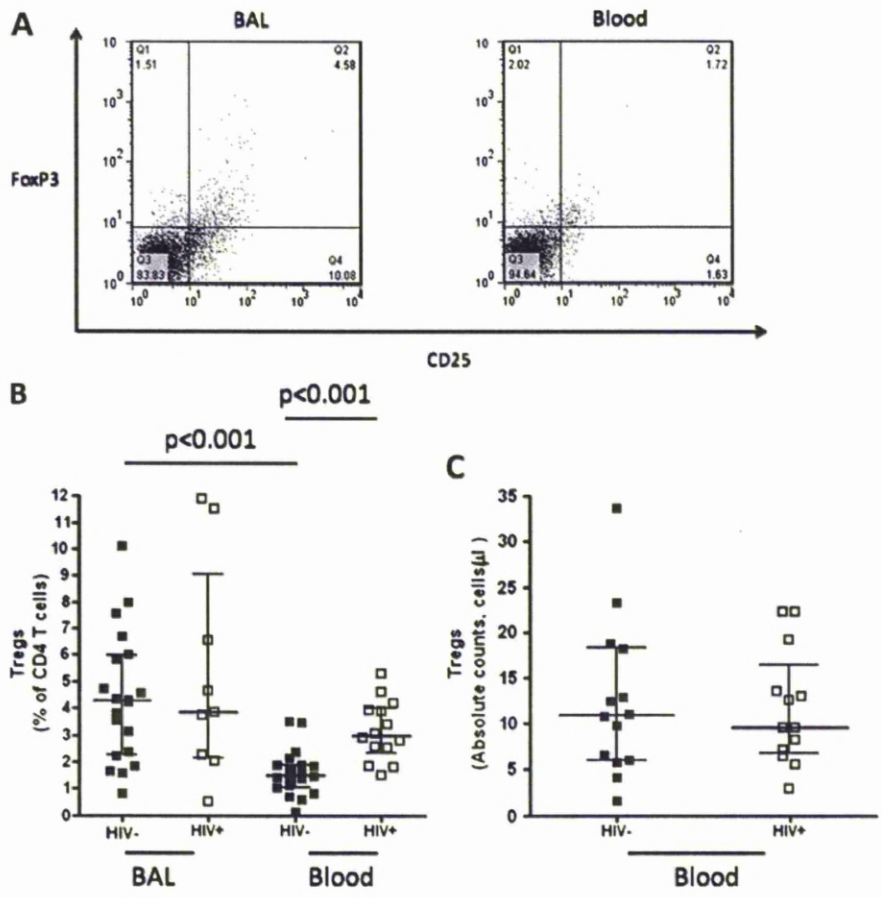

Figure 3 Higher frequency of regulatory T cells in BAL compared to peripheral blood, but altered in HIV-infected individuals. T lymphocytes obtained from BAL and peripheral blood were stained with anti-CD4 Pacific blue, anti-CD25 FITC and anti-Foxp3 PE fluorochrome conjugated antibodies. Regulatory T cells (Tregs) were defined as $CD4^+$ T cells expressing $CD25^{hi}$ and $FoxP3^+$. (A) A flow cytometry representative dot plot showing Tregs in BAL and peripheral blood from a healthy control. (B) The data shows a higher frequency of Tregs in BAL than peripheral blood in HIV-uninfected adults. It also shows a higher frequency of peripheral blood Tregs in HIV-infected individuals compared to HIV-uninfected adults. (C) The data shows no difference in the absolute counts of Tregs in peripheral blood between HIV-infected individuals and HIV-uninfected adults. Black horizontal bars represent median and IQRs. Statistical significance was analysed by the Mann–Whitney U test in the HIV-uninfected versus HIV-infected comparison, and Wilcoxon Signed rank test in the BAL versus peripheral blood comparison. p value <0.05 was used to determine statistical significance.

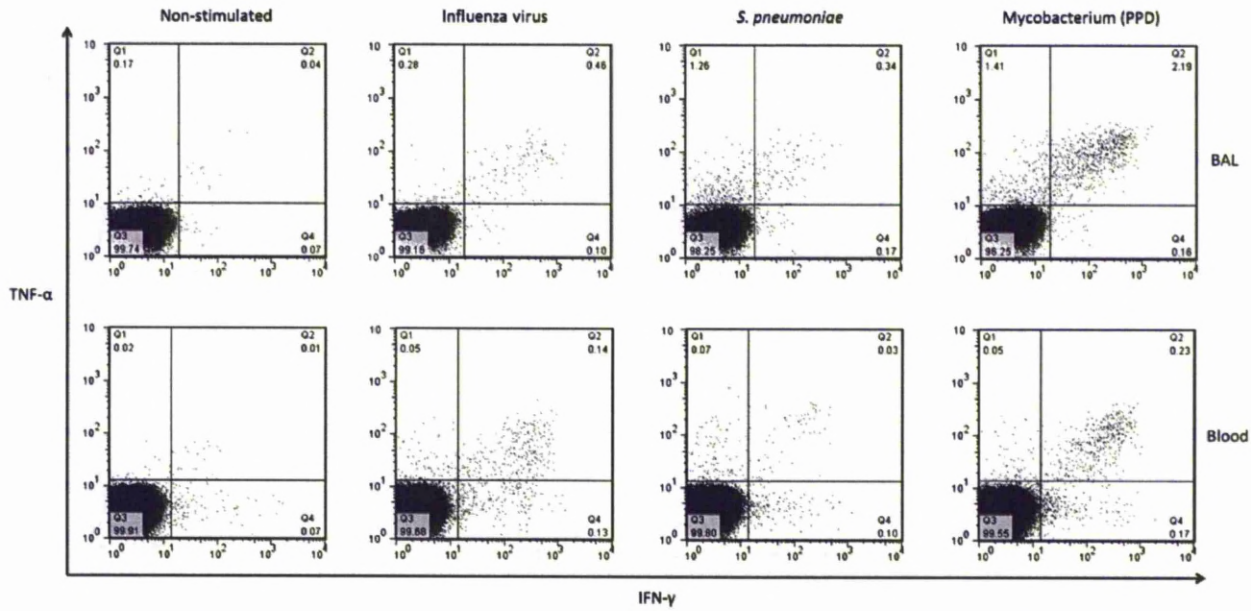

Figure 4 Representative flow cytometry dot flow from an HIV-uninfected adult showing multiple subsets of antigen-specific CD4$^+$ T cells in BAL and peripheral blood. BAL and peripheral blood lymphocytes were stimulated with antigens and T cell responses were measured by intracellular cytokine staining. Representative flow cytometry dot plots from an HIV-uninfected adult showing interferon-γ (IFN-γ) and TNF-alpha (TNF-α) responses in BAL (top) and peripheral blood (bottom) cells, in an unstimulated negative control and cells stimulated with influenza virus, *S. pneumoniae* and *M tuberculosis* antigens.

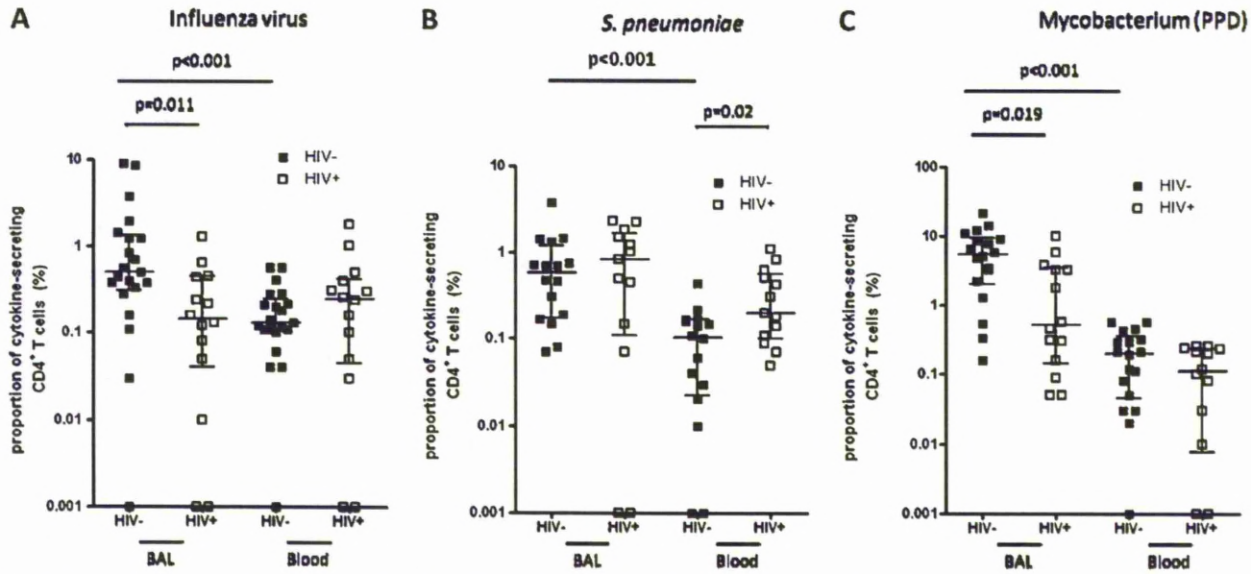

Figure 5 Lower frequency of antigen-specific BAL CD4$^+$ T cells in HIV-infected individuals compared to HIV-uninfected adults. BAL and peripheral blood lymphocytes were stimulated with antigens and the magnitude of the antigen-specific T cell response was measured by intracellular cytokine staining. The total of all cytokine-secreting CD4$^+$ T cells was used to represent the percentage frequency of antigen-specific cells. (A) The data shows a higher percentage frequency of influenza virus antigen-specific CD4$^+$ T cells in BAL compared to peripheral blood in HIV-uninfected adults. It also shows a lower percentage frequency of BAL influenza virus antigen-specific CD4$^+$ T cells in HIV-infected individuals compared to HIV-uninfected adults. (B) The data shows a higher percentage frequency of *S pneumoniae* antigen-specific CD4$^+$ T cells in BAL compared to peripheral blood in HIV-uninfected adults. It also shows a higher percentage frequency of *S pneumoniae* antigen-specific peripheral blood CD4$^+$ T cells in HIV-infected individuals compared to HIV-uninfected adults. (C) The data shows a higher percentage frequency of *M tuberculosis* antigen-specific CD4$^+$ T cells in BAL compared to peripheral blood in HIV-uninfected adults. It also shows a lower percentage frequency of BAL *M tuberculosis* antigen-specific CD4$^+$ T cells in HIV-infected individuals compared to HIV-uninfected adults. Black horizontal bars represent median and IQRs after background responses were subtracted from all the antigen-specific CD4 T cell responses. Statistical significance was analysed by the Mann–Whitney U test in the HIV-uninfected versus HIV-infected comparison, and Wilcoxon Signed rank test in the BAL versus peripheral blood comparison. p value <0.05 was used to determine statistical significance.

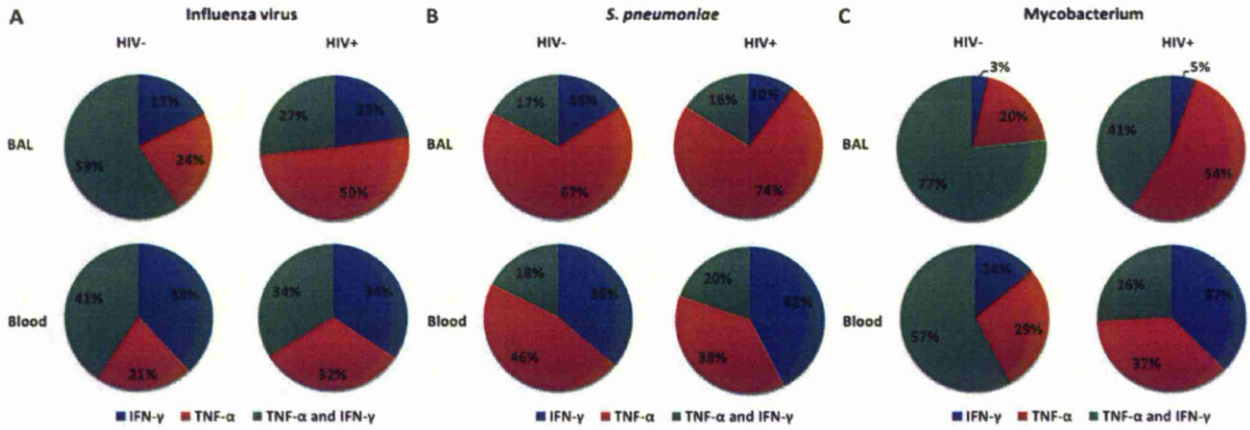

Figure 6 Lower proportion of polyfunctional antigen-specific CD4$^+$ T cells in BAL and peripheral blood of HIV-infected individuals compared to HIV-uninfected adults. BAL and peripheral blood lymphocytes were stimulated with antigens and the quality of the antigen-specific T cell response was measured by intracellular cytokine staining. The proportion of single producers (IFN-γ alone or TNF-α alone) and double producers (IFN-γ and TNF-α) in the antigen-specific CD4$^+$ T cell population was defined. (A) The data shows that in both BAL (upper) and peripheral blood (lower), the proportion of double producers (green) in the influenza virus antigen-specific CD4$^+$ T cell population was lower in HIV-infected individuals (right) than HIV-uninfected adults (left). It also shows that the proportion of subsets of antigen-specific CD4$^+$ T cells against influenza virus including IFN-γ single producers (blue), TNF-α single producers (red), and IFN-γ/TNF-α double producers (green) were different between BAL (upper) and peripheral blood (lower) in HIV-uninfected adults. (B) The data shows that the proportion of subsets of antigen-specific CD4$^+$ T cells against *S pneumoniae* (including single producers and double producers), were different between BAL (upper) and peripheral blood (lower) in HIV-uninfected adults. (C) The data shows that in BAL (upper) and peripheral blood (lower) the proportion of double producers (green) in the *M tuberculosis* antigen-specific CD4 T cell population was lower in HIV-infected individuals (right) than HIV-uninfected adults (left). It also shows that the proportion of subsets of antigen-specific CD4$^+$ T cells against *M tuberculosis* (including single producers, and double producers), were different between BAL (upper) and peripheral blood (lower) in HIV-uninfected adults.

responses were subtracted from all the antigen-specific CD4$^+$ T cell responses.

Further, in BAL and peripheral blood, there was a lower proportion of multiple cytokine-producing (polyfunctional) antigen-specific CD4$^+$ T cells against influenza virus (BAL, 59% vs 27%; Blood, 41% vs 34%) and *M tuberculosis* (BAL, median 77% vs 41%; Blood, median 57% vs 26%) in HIV-infected individuals compared to HIV-uninfected adults (figure 6A,C).

DISCUSSION

The authors have demonstrated compartment differences between T cell immunity in the bronchoalveolar space and the periphery. These include a predominant presence of effector memory T cells and regulatory CD4$^+$ T cells in BAL, and a higher percentage frequency of antigen-specific CD4$^+$ T cells against influenza virus, *S pneumoniae* and *M tuberculosis* in BAL compared to peripheral blood. Our data has also demonstrated that HIV-infected individuals have impaired pulmonary CD4$^+$ T cell immunity, which is characterised by lower proportions of total CD4$^+$ T cells and impaired antigen-specific BAL CD4$^+$ T cell response to influenza virus and *M tuberculosis* antigens.

Consistent with previous observations,[21] we noticed that BAL CD4$^+$ and CD8$^+$ T cells were predominantly of effector memory phenotype irrespective of HIV status, while peripheral blood T cell phenotypes were distributed among naive, central memory, effector memory and terminal effector. Effector memory T cells migrate to the lung following antigen presentation by antigen presenting cells in the local draining lymph nodes.[22] In the lung, effector memory T cells may be involved in mediating host defence against pathogens through macrophage activation and neutrophil recruitment.[9 10] Formulation of vaccines which can induce effector memory T cells in the lung, mimicking the natural route of exposure, might improve the efficacy of future vaccines against pulmonary mucosal infections such as pneumococcal pneumonia.

The authors observed a higher proportion of regulatory T cells in BAL fluid compared to peripheral blood in HIV-uninfected adults. Immunity in the lung is well regulated. A high degree of regulation is necessary because an excessive response may lead to destructive immunopathology, while a weak response may lead to prolonged infection. The higher proportion of Tregs in BAL, compared to peripheral blood in HIV-uninfected adults, may reflect the capacity for highly regulated immunity in the lung. While beneficial to the host, this sophisticated level of pulmonary immunocompetence poses a challenge to the potential success of mucosally-administered vaccines against respiratory pathogens.

In addition to the overall phenotypic differences between BAL and peripheral blood T cells seen in this study, the authors observed a higher percentage frequency of antigen-specific CD4$^+$ T cells against influenza virus, *S pneumoniae* and *M tuberculosis* in BAL, when compared to the peripheral blood of HIV-uninfected adults. The authors investigated a variety of respiratory antigens in this study and demonstrated that each pathogen has its unique cytokine secreting profile. The influenza virus antigen-specific CD4$^+$ T cells were likely induced through previous infection or exposure. The pneumococcal antigen-specific CD4$^+$ T cells were probably induced through previous exposure following contact, colonisation in the nasopharynx or disease. The *M tuberculosis* antigen-specific CD4$^+$ T cells may have been induced by previous BCG vaccination, latent infection or exposure. Despite differences in kinetics of exposure, our data suggest that antigen-specific CD4$^+$ T cell responses against common respiratory pathogens are compartmentalised in the lung, where they may confer protection at the site of antigen entry. Therefore, the induction of pulmonary antigen-specific CD4$^+$ T cells

through vaccination may provide a useful strategy for preventing respiratory infections in the lung.

In the context of HIV, first, the authors observed a higher proportion of BAL and peripheral blood effector memory $CD4^+$ and $CD8^+$ T cells in HIV-infected individuals than in HIV-uninfected adults. This may be attributed to the high level of hyper-activation associated with HIV infection,[23] whereby naive T cells are driven along their differentiation pathway to become effector memory T cells.

Second, the authors observed a higher proportion of Tregs in the blood of HIV-infected individuals compared to HIV-uninfected adults. This observation is consistent with other studies on Tregs in peripheral blood during HIV infection.[24] Expressing Tregs as absolute counts, however, showed that there was no difference between HIV-uninfected adults and HIV-infected individuals. This suggests that, during HIV infection, non-Tregs are preferentially depleted compared to Tregs. In contrast, the authors did not observe a difference in proportion of BAL Tregs between HIV-infected individuals and HIV-uninfected adults. However, the proportion of BAL $CD8^+$ T cells was higher in HIV-infected compared to HIV-uninfected adults, suggesting that there is either a depletion of $CD4^+$ T cells or an infiltration of $CD8^+$ T cells. Taking these observations together, there is likely to be an altered ratio of Tregs to effector $CD4^+$ and $CD8^+$ T cells in BAL. This factor may either tip the balance to over-regulation of CD4 responses or lack of control of CD8 infiltrates in HIV-infected adults.

Third, consistent with the earlier observation on altered proportions of pulmonary T cell subsets in HIV-infected individuals, the percentage frequencies of influenza virus and *M tuberculosis* antigen-specific BAL $CD4^+$ T cells were lower in HIV-infected individuals compared to HIV-uninfected adults. This is in line with a recent observation that there was depletion of BAL *M tuberculosis* antigen-specific $CD4^+$ T cells in HIV-infected individuals.[21] Recent work from other investigators has shown a preferential depletion of peripheral blood *M tuberculosis* antigen-specific but not cytomegalovirus (CMV) antigen-specific $CD4^+$ T cells in HIV-infected adults.[25] They concluded that *M tuberculosis* antigen-specific adaptive immunity is particularly vulnerable to HIV-associated immune damage. This study has shown that adaptive immunity to other respiratory antigens such influenza virus and not only *M tuberculosis* is also impaired in HIV-infected individuals. The impaired antigen-specific BAL $CD4^+$ T cell response observed in this study might help to explain the increased susceptibility of HIV-infected individuals to influenza virus and *M tuberculosis* infections.[17 26] The impaired BAL $CD4^+$ T cell immunity to influenza virus in HIV-infected adults may result in increased risk to secondary bacterial infection such as pneumococcal pneumonia. The authors observation that there were detectable antigen-specific $CD4^+$ T cell defects in BAL, but not in peripheral blood, has implications for clinical practice and for vaccine development. Most available assays, being based on peripheral blood, do not detect local responses in the lung and may therefore give misleading results when used for diagnosis or for estimations of vaccine efficacy.

Lastly, the authors demonstrated that there was a lower proportion of polyfunctional $CD4^+$ T cells in BAL and peripheral blood of HIV-infected individuals compared to HIV-uninfected adults. This observation is consistent with others that have showed impaired polyfunctionality in *M tuberculosis* antigen-specific $CD4^+$ T cells in BAL from HIV-infected adults.[21] Recently, it has been shown that during HIV infection, *M tuberculosis* antigen-specific T cells in blood change from polyfunctional $CD4^+$ T cells to a more predominant monofunctional $CD8^+$ T cell phenotype.[27] The authors speculate that owing to hyperactivation induced by HIV,[23] T cells are driven towards the terminal differentiation stages of their life cycle, where there is loss of the ability to secrete more than one cytokine.[28] It has been reported that polyfunctional T cells may be functionally superior to monofunctional T cells and may provide a correlate of protection against parasitic,[29] bacterial[30] and viral pathogens.[31] This finding provides supporting evidence to the suggestion that an impaired repertoire of multifunctionality, as well as lower absolute numbers of respiratory antigen-specific $CD4^+$ T cell immunity, may contribute to the high burden of respiratory diseases in HIV-infected individuals.

In conclusion, the authors have demonstrated that HIV infection is associated with impaired pulmonary antigen-specific $CD4^+$ T cell immunity against viral and bacterial respiratory pathogens. These defects in pulmonary immunity may help to explain the observed increased risk of acute lower respiratory tract infections in HIV-infected adults at any point during the fall in their systemic CD4 count. A greater understanding of antigen-specific T cell responses that occur in the lungs of HIV-infected individuals will potentially enable better planning of clinical care for these individuals – in terms of prophylactic treatments against common infectious pathogens and the timing of anti-retroviral medication. In addition, it will also provide appropriate markers of efficacy in future vaccine trials for common respiratory pathogens such as *S pneumoniae*, *M tuberculosis* and influenza virus.

Acknowledgements The authors would like to thank the volunteers and the staff of the Queen Elizabeth Central Hospital and MLW diagnostic laboratory, Blantyre, Malawi, for their willing cooperation with this study. We thank Professor Malcolm Molyneux for proof reading the manuscript. We thank Anstead Kankwatira and Rose Malamba for their clinical support, and Visopo Harawa for the laboratory support. We thank James Paton for kindly providing the pneumococcal strains. We acknowledge the support of the Malawi-Liverpool-Wellcome Trust Clinical Research Programme, NIHR Biomedical Research Centre in Microbial Diseases, Royal Liverpool University Hospital and the NWDA for infrastructural support.

Funding This work was supported by a grant to K.C.J from the Commonwealth Scholarship Commission (UK), and grants to S.B.G and to R.S.H. from the Wellcome Trust (UK).

Competing interests None.

Ethics approval This study was conducted with the approval of the College of Medicine Research and Ethics Committee (Malawi) and the Liverpool School of Tropical Medicine Research Ethics Committee (United Kingdom).

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Mathers C,** Boerma T, Ma Fat D. *The Global Burden of Disease: 2004 Update*. Geneva, Switzerland: World Health Organisation, 2008.
2. **Mukadi YD,** Maher D, Harries A. Tuberculosis case fatality rates in high HIV prevalence populations in sub-Saharan Africa. *AIDS* 2001;**15**:143–52.
3. **Turner D.** Improving Global Health by Preventing Pneumococcal Disease. In: APPG, ed. London, United Kingdom: UK Government, 2008:1–35.
4. **UNAIDS.** *AIDS Epidemic Update: November 2009*. Geneva, Switzerland: UNAIDS WHO, 2009.
5. **van der Poll T,** Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. *Lancet* 2009;**374**:1543–56.
6. **Cooper AM.** Cell-mediated immune responses in tuberculosis. *Annu Rev Immunol* 2009;**27**:393–422.
7. **Malley R.** Antibody and cell-mediated immunity to *Streptococcus pneumoniae*: implications for vaccine development. *J Mol Med* 2010;**88**:135–42.
8. **Thomas PG,** Keating R, Hulse-Post DJ, *et al*. Cell-mediated protection in influenza infection. *Emerg Infect Dis* 2006;**12**:48–54.
9. **Dalton DK,** Pitts-Meek S, Keshav S, *et al*. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* 1993;**259**:1739–42.
10. **Laan M,** Cui ZH, Hoshino H, *et al*. Neutrophil recruitment by human IL-17 Via C-X-C chemokine release in the airways. *J Immunol* 1999;**162**:2347–52.
11. **Scriba TJ,** Kalsdorf B, Abrahams DA, *et al*. Distinct, specific IL-17- and IL-22-producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. *J Immunol* 2008;**180**:1962–70.

12. **Wilson EB,** Livingstone AM. Cutting edge: CD4+ T cell-derived IL-2 is essential for help-dependent primary CD8+ T cell responses. *J Immunol* 2008;**181**:7445–8.
13. **de Bree GJ,** Daniels H, Schilfgaarde M, *et al*. Characterization of CD4+ memory T cell responses directed against common respiratory pathogens in peripheral blood and lung. *J Infect Dis* 2007;**195**:1718–25.
14. **Barry SM,** Lipman MC, Bannister B, *et al*. Purified protein derivative-activated type 1 cytokine-producing CD4+ T lymphocytes in the lung: a characteristic feature of active pulmonary and nonpulmonary tuberculosis. *J Infect Dis* 2003;**187**:243–50.
15. **Brenchley JM,** Knox KS, Asher AI, *et al*. High frequencies of polyfunctional HIV-specific T cells are associated with preservation of mucosal CD4 T cells in bronchoalveolar lavage. *Mucosal Immunol* 2008;**1**:49–58.
16. **Jordano Q,** Falcón V, Almirante B, *et al*. Invasive pneumococcal disease in patients infected with HIV: still a threat in the era of highly active antiretroviral therapy. *Clin Infect Dis* 2004;**38**:1623–8.
17. **Sonnenberg P,** Glynn JR, Fielding K, *et al*. How soon after infection with HIV does the risk of tuberculosis start to increase? A retrospective cohort study in South African gold miners. *J Infect Dis* 2005;**191**:150–8.
18. **Gordon SB,** Malamba R, Mthunthama N, *et al*. Inhaled delivery of 23-valent pneumococcal polysaccharide vaccine does not result in enhanced pulmonary mucosal immunoglobulin responses. *Vaccine* 2008;**26**:5400–6.
19. **Sallusto F,** Lenig D, Förster R, *et al*. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999;**401**:708–12.
20. **Fontenot JD,** Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003;**4**:330–6.
21. **Kalsdorf B,** Scriba TJ, Wood K, *et al*. HIV-1 infection impairs the bronchoalveolar T-cell response to mycobacteria. *Am J Respir Crit Care Med* 2009;**180**:1262–70.
22. **Bakocević N,** Worbs T, Davalos-Misslitz A, *et al*. T cell-dendritic cell interaction dynamics during the induction of respiratory tolerance and immunity. *J Immunol* 2010;**184**:1317–27.
23. **Hazenberg MD,** Otto SA, van Benthem BH, *et al*. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS* 2003;**17**:1881–8.
24. **Kolte L,** Gaardbo JC, Skogstrand K, *et al*. Increased levels of regulatory T cells (Tregs) in human immunodeficiency virus-infected patients after 5 years of highly active anti-retroviral therapy may be due to increased thymic production of naive Tregs. *Clin Exp Immunol* 2009;**155**:44–52.
25. **Geldmacher C,** Ngwenyama N, Schuetz A, *et al*. Preferential infection and depletion of *Mycobacterium tuberculosis*-specific CD4 T cells after HIV-1 infection. *J Exp Med* 2010;**207**:2869–81.
26. **Lin JC,** Nichol KL. Excess mortality due to pneumonia or influenza during influenza seasons among persons with acquired immunodeficiency syndrome. *Arch Intern Med* 2001;**161**:441–6.
27. **Sutherland JS,** Young JM, Peterson KL, *et al*. Polyfunctional CD4(+) and CD8(+) T cell responses to tuberculosis antigens in HIV-1-infected patients before and after anti-retroviral treatment. *J Immunol* 2010;**184**:6537–44.
28. **Seder RA,** Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* 2008;**8**:247–58.
29. **Darrah PA,** Patel DT, De Luca PM, *et al*. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* 2007;**13**:843–50.
30. **Forbes EK,** Sander C, Ronan EO, *et al*. Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against *Mycobacterium tuberculosis* aerosol challenge in mice. *J Immunol* 2008;**181**:4955–64.
31. **Nebbia G,** Mattes FM, Smith C, *et al*. Polyfunctional cytomegalovirus-specific CD4+ and pp65 CD8+ T cells protect against high-level replication after liver transplantation. *Am J Transplant* 2008;**8**:2590–9.

Journal club

MRSA colonisation may lead to increased mortality in patients with cystic fibrosis

The long-term impact of respiratory tract methicillin-resistant *Staphylococcus aureus* (MRSA) on cystic fibrosis (CF) outcomes is unclear. This cohort study used longitudinal data from the US CF registry between 1996 and 2008. The aim of the study was to assess whether acquisition of MRSA influenced mortality.

A total of 19 833 MRSA negative patients aged 6–45 years were included in the study. Over the study period 5759 patients developed positive cultures for MRSA and there were 2537 deaths. The mortality rate was 27.7 deaths (95% CI 25.3 to 30.4) per 1000 patient-years in patients with MRSA and 18.3 deaths (95% CI 17.5 to 19.1) per 1000 patient-years in those without MRSA. After adjusting for variables associated with disease severity, the risk of death in patients positive for MRSA was higher than in patients who never cultured MRSA, with the greatest risk in patients chronically colonised with MRSA. Interestingly, patients who cleared MRSA within a year did not have an increased risk of death. No treatment or eradication data were described in the study.

This study provides convincing evidence that MRSA is an important pathogen in the CF airway. Questions remain about both the pathogenesis of MRSA infection and the optimal medical management in chronically infected patients. For now it is essential that strict infection control is in place to minimise transmission, and that eradication protocols are followed after a new identification.

► **Dasenbrook EC**, Checkley W, Merlo CA, *et al*. Association between respiratory tract methicillin-resistant *Staphylococcus aureus* and survival in cystic fibrosis. *JAMA* 2010;**303**:2386–92.

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Published Online First 21 October 2010

Thorax 2011;**66**:382. doi:10.1136/thx.2010.151290